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(54) Title: TARGET BINDING POLYPEPTIDE

(57) Abstract

A target-binding polypeptide having (a) a stable core polypeptide region (SCR); and (b) at least one target-binding region (TBR), in which the target-binding region(s) are covalently attached to the SCR and which have optionally been subjected to a maturation step in order to modify the specificity, the affinity or the avidity of binding to the target. The polypeptides may self associate to form stable dimers, aggregates or arrays. The polypeptides of the invention have utility in the diagnostic, therapeutic, predictive or preventative fields of the pharmaceutical and health care industries, as well as more general application in the detection and assay of chemical entities.

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TARGET BINDING POLYPEPTIDE

This invention relates to the construction, application and production of novel polypeptides with enhanced or modified binding activity or specificity to haptens and antigens.

The invention also relates to the construction, modification and selection of recombinant antibody-like molecules derived from expression of libraries of surface presenting antigen- or hapten-binding moieties, and to uses of these molecules.

The polypeptides of the invention have utility in the diagnostic, therapeutic, predictive or preventative fields of the pharmaceutical and health care industries, as well as more general application in the detection and assay of chemical entities.

Background of the Invention

Antibodies are protein molecules which possess a binding affinity for a target antigen or hapten. Due to the specificity of the binding interaction, antibodies are commonly used as diagnostic and therapeutic reagents. Monoclonal antibodies are derived from a pure cell line such as hybridoma cells; however, the hybridoma technology is expensive, time-consuming to maintain and limited in scope. It is not possible to produce monoclonal antibodies, much less antibodies of the appropriate affinity, to a complete range of antigens.

Antibody genes or fragments thereof can be cloned and expressed in *E. coli* in a biologically functional form. Antibodies and antibody fragments can also be produced by recombinant DNA technology using either bacterial or mammalian cells. In the Fab region of an antibody, the combination of the two heavy and light chains provides six variable surface loops at the extremity of the molecule.

These loops in the outer domain (Fv) are termed complementarity-determining-regions (CDRs), and provide the specificity of binding of the antibody to its antigenic target. This binding function is localised to the variable domains of the antibody molecule, which are located at the amino-terminal end of both the heavy and light chains. This is illustrated in Figure 1. The variable regions of some antibodies remain non-covalently associated (as V_HV_L dimers, termed Fv regions) even after proteolytic cleavage from the native antibody molecule, and retain much of their antigen recognition and binding capabilities. Methods of manufacture of two-chain Fv substantially free of constant region are disclosed in US-4,642,334

Recombinant Fv fragments are prone to dissociation, and therefore some workers have chosen to covalently link the two domains to form a construct designated scFv, in which two peptides with binding domains (usually antibody heavy and light variable regions) are joined by a linker peptide connecting the C-terminus of one domain to the N-terminus of the other, so that the relative positions of the antigen binding domains are consistent with those found in the original antibody (see Figure 1).

Methods of manufacture of covalently linked Fv fragments are disclosed in US-4,946,778 and US-5,132,405. Further heterogeneity can be achieved by the production of bifunctional and multifunctional agents (Huston *et al* U.S. Patent No. 5,091,513, and Ladner *et al* U.S. Patent No. 4,816,397).

The construction of scFv libraries is disclosed for example in European Patent Application No. 239400 and U.S. Patent No.4,946,778. However, single-chain Fv libraries are limited in size because of problems inherent in the cloning of a single DNA molecule encoding the scFv. Non-scFv libraries, such as V_H or Fab libraries, are also

known, (Ladner and Guterman WO 90/02809), and may be used with a phage system for surface expression (Ladner *et al* WO 88/06630 and Bonnert *et al* at WO 92/01047).

For use in antibody therapy, monoclonal antibodies, which are usually of mouse origin, have limited use unless they are first "humanised", because they elicit an antigenic response on administration to humans. The variable domains of an antibody consist of a β-sheet framework with six hypervariable regions (CDRs) which fashion the antigen-binding site. Humanisation consists of substituting mouse sequences that provide the binding affinity, particularly the CDR loop sequences, into a human variable domain structure. The murine CDR loop regions can therefore provide the binding affinities for the required antigen. Recombinant antibody "humanisation" by grafting of CDRs is disclosed by Winter *et al* (EP-239400).

The expression of diverse recombinant human antibodies by the use of expression/combinatorial systems has been described. (Marks *et al*, J. Mol. Biol. 1991 <u>222</u> 581-597). Recent developments in methods for the expression of peptides and proteins on the surface of filamentous phage (McCafferty *et al*, Nature 1991 <u>348</u> 552; Clackson *et al*, J. Mol. Biol., 1991 <u>352</u> 624-28) offer the potential for the selection, improvement and development of these reagents as diagnostics and therapeutics. The use of modified bacteriophage genomes for the expression, presentation and pairing of cloned heavy and light chain genes of both mouse and human origins has been described (Hoogenboom *et al*, Nucl. Acids. Res., <u>19</u> 4133-4137; Marks *et al* 1991 *op.cit.* and Bonnert *et al*, WPI Acc. No. 92-056862/07)

Receptor molecules, whose expression is the result of the receptor-coding gene library in the expressing organism, may also be displayed in the same way

(Lerner and Sorge, WO 90/14430). The cell surface expression of single chain antibody domains fused to a cell surface protein is disclosed by Ladner *et al* WO 88/06630.

Affinity maturation is a process whereby the binding specificity, affinity or avidity of an antibody can be modified. A number of laboratory techniques have been devised whereby amino acid sequence diversity is created by the application of various mutation strategies, either on the entire antibody fragment or on selected regions such as the CDRs. Mutation to change enzyme specific activity has also been reported. The person skilled in the art will be aware of a variety of methods for achieving random or site-directed mutagenesis, and for selecting molecules with a desired modification. Mechanisms to increase diversity and to select specific antibodies by the so called "chain shuffling" technique, ie. the reassortment of a library of one chain type e.g. heavy chain, with a fixed complementary chain, such as light chain, have also been described (Kang *et al*, Proc. Natl. Acad. Sci. USA, 1991 88 4363-466; Hoogenboom *et al*, Nucl. Acid Res., 1991 19 4133-4137; Marks *et al*, Bio/Technology, 1992 10 779-783).

In order to overcome the problems of human reactions to murine sequences in any part of the V-domains, framework or constant regions of the antibodies, recombinant human antibody-gene libraries may be constructed from a variety of human tissues, including peripheral blood lymphocytes (Winter and Milstein Nature, 1991 349 293). Adult humans will already have been subjected to antigenic stimulation, and therefore the capacity of the pre-immunised adult B-cell population to recognise as wide a range of antigens is diminished compared to the naive B-cell population, and is reflected in the restricted populations of antibody mRNA molecules.

Thus in order to access as wide a range of antigen-binding potential as possible, one of the tissues of choice is foetal peripheral blood, which being naive has a higher proportion of IgM antibody molecules than adult blood, (approximately 70% compared to the 30% for IgG), and provides the ideal source of genetic material for the construction of an antibody library destined for maturation (evolution) to a breadth where a wide range of antigens can be bound.

Summary of the Invention

The present invention therefore includes within its scope:

1) The identification and construction of novel recombinant target binding polypeptides;

2) Modification of such reagents to alter their performance, for example by mechanisms involving the mutation of their DNA coding regions; and

3) Further changing these reagents either at the genetic or the protein level, by reassortment of their subcomponents.

According to a first aspect, the invention provides a target-binding polypeptide having:

a) a stable core polypeptide region (SCR); and

b) at least one target-binding region (TBR).

in which the target-binding region(s) has optionally been subjected to a maturation step in order to modify the specificity, the affinity or the avidity of binding to the target.

We have been able to design and construct polypeptides according to the invention in which the specificity, affinity or avidity of binding is modified,

without the necessity for performing a maturation step. For example this has been done using immunoglobulin (Ig) and CD8.

We describe the construction of monovalent target binding polypeptides in which the TBR is covalently linked to a SCR. The SCR is preferably formed by association of two covalently linked Ig-like domains of the Ig superfamily such as to antibody variable domains or CD8 domains.

We also show how polyfunctional target binding polypeptides can be produced by forming separate or overlapping TBRs on a SCR. We have also shown that the Ig-like domains of members of the Ig superfamily can be constructed as SCRs and joined non-covalently to produce bifunctional or polyfunctional target-binding polypeptides. We describe how to design amino acid sequences which can covalently link Ig-like domains and thereby direct self association to form stable dimers, aggregates or arrays preferably with bifunctional or polyfunctional specificity.

The target-binding region is able to bind a target molecule, which may be a chemical entity of any type. For example, the target may be a small molecule such as a pesticide or a drug, a hormone such as a steroid, an amino acid, a peptide, or a polypeptide; an antigen, such as a bacterial, viral or cell surface antigen; antibodies or other members of the Ig superfamily; a tumour marker, a growth factor, etc. The skilled person will readily be able to select a wide variety of targets of interest.

Where the polypeptide of the invention is to be used for *in vitro* diagnostic purposes, the core polypeptide region may be any suitable protein. However, where the polypeptide of the invention is intended for use *in vivo*, the core polypeptide region should preferably be non-antigenic. Thus any normal human protein of the type which is present in serum or displayed on cell surfaces, and is

generally tolerated, would be suitable. Certain domains of normal cell-surface proteins can be produced in soluble form and, by the methods of this invention, have their affinity properties enhanced or modified. For human proteins which have as their natural target T-cell surface proteins, the soluble fragments become potential immunomodulatory therapeutic reagents especially useful for transplantation. Many of these fragments will possess homology to proteins of the immunoglobulin superfamily.

In particularly preferred embodiments of the invention, the target is selected from the group consisting of glycophorin or other red blood cell surface proteins, influenza virus neuraminidase; viral antigens such as hepatitis B antigen, and the gp40 protein of HIV; tumour markers, cell surface proteins such as CD28 and CD4; transforming growth factor α (TGF-α); and leukaemia inhibitory factor (LIF). For both diagnostic and therapeutic applications, it is particularly useful if the target binding region has more than one specificity. It is especially preferred that the target binding polypeptide possesses affinity to more than one target; this affinity is provided by separate or overlapping surfaces, thus forming a bifunctional or polyfunctional reagent. It is envisaged that bifunctional or polyfunctional reagents can also be formed by covalent or non-covalent attachment of individual target binding polypeptides, optionally using a linker polypeptide.

In a second aspect, the invention provides a DNA construct encoding the target binding polypeptide.

In a third aspect, the invention provides a method for producing a DNA construct encoding a target binding polypeptide of the invention, comprising the step of subjecting DNA encoding a target binding polypeptide to one or more cycles of mutagenesis and selection to obtain a

sub-population of DNA molecules encoding target binding polypeptides having modified characteristics of affinity, specificity, or avidity.

Preferably the DNA encoding the target binding polypeptide is present in a replication-competent element or display vector, ie. a vector which is self-replicating, optionally when present in a suitable host. The display vector is preferably selected from the group consisting of bacteriophage, filamentous bacteriophages such as Fd, viruses, bacteria, yeast, slime moulds, or mammalian cells.

Mutagenesis may be either random or site-directed, and the person skilled in the art will be aware of many suitable methods for carrying out this step. One or more target binding regions of the target binding polypeptide may be subjected to mutagenesis.

A preferred mutation system for use in the invention utilises specific mutator strains of *Escherichia coli*, designated *mutD* and *mutT1* (R. Fowler *et al*, J. Bacteriol., 1986 <u>167</u> 130). These particular mutator strains permit transfection with phage, making them especially useful for the purposes of the invention.

In a preferred embodiment, the method of producing the target binding polypeptide comprises the steps of:

a) isolating DNA encoding the framework structure of one or more desired target-binding polypeptides by means of the polymerase chain reaction;

b) optionally subjecting the DNA to mutagenesis in order to induce mutations in one or more target binding regions of the target binding polypeptide;

c) inserting the DNA into one or more display vectors;

d) selecting a sub-population of display vectors displaying target binding polypeptides of desired specificity, avidity or affinity;

e) subjecting the selected sub-population to one or more cycles of mutagenesis and selection in order to obtain a sub-population of display vectors displaying target binding polypeptides having modified characteristics of affinity, specificity or avidity; and

f) inserting DNA encoding the modified target binding polypeptides into a high level expression vector.

Selection of the sub-population of display vectors may be achieved by a variety of conventional methods such as target binding, fluorescence-activated cell sorting, or exploitation of the biotin-avidin or biotin-streptavidin systems. A particularly preferred method is affinity selection on an insoluble support such as immunotubes; this has been found to be especially convenient.

It will be appreciated that the invention therefore also provides a method of producing the target binding polypeptide, by transferring the high level expression vector described above into an appropriate expression host, expressing the target binding polypeptide, and isolating the protein thus produced.

It will also be clearly understood that the target binding regions and the stable core polypeptide may be different regions of the same molecule, or may be derived from different molecules.

Types of target binding polypeptide constructs which are contemplated by the invention include modified

antibodies or antibody fragments, scFv fragments comprising an association link to permit continuous reassortment, modified CD8 molecules, for example single chain CD8, and combinations of antibody molecules or fragments thereof with CD8 or other molecules related to the immunoglobulin superfamily such as the individual domains of the MHC Class I and II molecules. For example, the α3 domain of MHC Class I binds to CD8, and therefore soluble versions of α3 become potential immunomodulatory reagents. Preferred constructs utilizing CD8 include:

a) Single-chain molecules in which the V-like domains only of the α and β subunits are linked, and

b) Molecules in which the N-terminal amino acid has been altered from lysine to serine, in order to alter the charge balance of the signal peptides, thus enabling bacterial expression without adversely affecting biological activity.

Target binding polypeptides may include covalently attached polypeptide tails which can be TBRs or which may permit non-covalent association to other TBPs.

Although the following description refers in some examples specifically to IgG type antibodies and their fragments, it will be clearly understood that the invention is also applicable to other types of antibody molecule, such as IgM and IgA.

The DNA sequence encoding the target binding polypeptide may be cloned into any vector which will allow display of the polypeptide on bacteriophage or cell surfaces. Preferred vectors include pHFA, whose construction is described in International Patent Application No. PCT/AU93/00228, and its structure is illustrated in Figure 4. Preferred bacterial hosts for protein expression are *E. coli* and *Bacillus subtilis*.

Detailed Description of the Drawings

The invention will be described in detail by reference to the following non-limiting examples and to the drawings, in which:

Figure 1 illustrates the structure of antibodies and their fragments:

a) This shows the polypeptide chain structure of a typical IgG antibody molecule, which is composed of two identical heavy and two identical light chains, each divided into variable (V) and constant (C) domains. The whole IgG molecule has two identical antigen binding surfaces termed Fv regions, which are formed by the pairing of V_H and V_L chains. The combination of V_H and V_L provides 6 loops, termed complementarity-determining regions (CDRs), at the extremity of the molecule and these provide the antigen binding surface and thereby the binding specificity of the antibody to its target antigen.

b) an Fab antibody fragment comprises one light and a portion of one heavy chain.

c) A single-chain scFv is shown as V_H and V_L domains joined by a peptide linker between the C-terminus of V_H to the N-terminus of V_L. Both Fab and Fv fragments are expected to have the same antigen binding surface as the parent antibody.

Figure 2 shows antibody fragments such as Fab and scFv molecules displayed on the surface of filamentous Fd bacteriophage by covalent fusion to either the minor coat protein at the tip of the phage, the gene III protein or as fusions with the major, gene VIII coat protein. For display of Fab molecules, only one of the chains (Heavy or Light) is anchored to the phage coat protein, and the other chain is provided in soluble form in the host cell periplasm. The Fd bacteriophage are still viable, although fusions on the gene III protein reduce infectivity into

host cells.

Figure 3 shows how pools (libraries) of heavy and light variable chains can be constructed into a Fd phage display vector with one of the chains fused to either the gene III protein or gene VIII protein of the phage. The display vector is transfected into host cells to generate a dual-combinatorial library. Each host cell produces viable Fd phage in which the antibody fragment is displayed on the phage surface and the gene encoding the antibody is packaged with the viral genome. Affinity purification of the phage is based on affinity to a target antigen, and allows simultaneous recovery of the gene encoding the antibody from the viable phage. Phagemid display vectors can improve transformation yields, but require helper phage to assemble viable progeny. Alternative strategies include the construction of hierarchical libraries in which one chain is held constant and displayed with a library of the second chain to select the highest affinity paired chains. More complex libraries can be constructed using gene pools on separate display vectors and then cross-transfecting host cells. Gene recovery will depend on the relative packing efficiency of the two vectors.

Figure 4 shows the structure of the phagemid vector pHFA. This vector has the ability in suppressor strains of *E. coli* to express cloned antibodies as fusions with the gene III protein on the surface of the Fd phage, whereas in non-suppressor strains the cloned antibody genes are expressed as soluble products. The lacZ promoter allows induction of expression with IPTG, and the FLAG tail, which is expressed as a fusion with the antibody, is used for detection of synthesis and affinity purification.

Figure 5 shows the series of scFv NC10 deletion linker constructs and the theoretical minimum distance (in

Angstroms) spanned by the polypeptide linker.

Figure 6 shows the DNA sequence of synthetic oligonucleotide duplexes encoding peptide linkers of different lengths that were inserted into appropriately restricted pPOW-scFv NC10.

Figure 7 shows the analysis of synthesised scFv NC10 proteins from the V_H.15.V_L, V_H.10.V_L, V_H.5.V_L and V_H.V_L from uninduced (lanes 1-4) and induced (lanes 5-8) respectively on a Coomassie gel (upper panel) and a Western blot (lanes 9-16 lower panel) of the Coomassie gel probed with the anti-FLAG, M2 antibody (IBI, New Haven, CT) followed by goat anti-mouse horse radish peroxidase conjugate (Sigma) as the second antibody and detected by enhanced chemiluminescence (Amersham).

Figure 8 shows a summary of observed scFv NC10 associations and activity to its target antigen.

Figure 9 shows the similarity in structure between an antibody Fv fragment and a CD8 α chain heterodimer.

a) This is a ribbon drawing of a V_H or V_L molecule showing the CDR loops numbered 1-6 and the structurally conserved framework regions as ribbons.

b) This is a ribbon drawing of two CD8 α chains. The regions corresponding to antibody CDR loops are shown at the top of the molecule and numbered.

The homodimer is oriented with the molecular dyad axis situated vertically in the plane of the page. CVR-like loops from the top surface of the molecule as shown, and the CDR 1-like, CDR 2-like and CDR 3-like loops are labelled 1, 2 and 3 respectively for one sub-unit, and 1', 2' and 3' for the other sub-unit. The C-termine extend from the bottom of the molecule. The loops forming the dimer interface are the CDR 3-like loops (top) and C-C' loops (bottom).

Figure 10 shows a Coomassie stained SDS-PAGE gel of the synthesised scCD8 in pPOW using *E. coli* host cells pop2136 showing whole cell lysates. Arrows show the

positions of the fused and mature (cleaved signal sequence) scCD8. In this figure:

Lane 1	uninduced cells containing pPOW-scCD8
Lane 2	4 hours post-induction of pPOW-scCD8
Lane 3	uninduced cells containing pPOW-Lys→Ser scCD8
Lane 4	4 hours post-induction of pPOW-Lys→Ser scCD8
Lane 5	pre-stained molecular weight markers.

Figure 11 illustrates a scheme whereby individual antibody genes can be affinity matured. Individual genes can be selected from phage display libraries, and then subjected to rounds of *in vivo* or *in vitro* mutations. The affinity matured antibody fragments are then selected for their ability to bind antigen, prior to further rounds of mutation or high level gene expression. Entire antibody libraries can be increased in complexity by cyclic rounds of mutation prior to selection of individual phage via panning or affinity selection.

Figure 12 shows results of ELISA screening of colonies subjected to mutation for affinity maturation.

Figure 13 shows an example of the p569 vector (a gift from W. Nellen), a shuttle vector for expression in *D. discoideum*. The vector has the alpha L fucosidase promoter and signal sequence, a multi-cloning site, a transcription terminator and the transposon Tn903 for selection by G418.

Figure 14 shows the tertiary structure depicted as the polypeptide backbone of NC10 scFv fragments complexed with two influenza neuraminidase subunits solved at 3 Angstroms resolution by X-ray diffraction analysis. The linker polypeptide joining the heavy and light chain variable regions is not depicted in this figure. In the crystal structure two Fv fragments are associated back-to-back to dimerise two different neuraminidase subunits. In the context of the scFv fragments the dimeric Fv module can be considered a bifunctional reagent.

Figure 15 shows a model of two neuraminidase tetramers which are bound together by four NC10 scFv dimers in solution as resolved by electron microscopy.

Figure 16 shows a model of bifunctional Fv molecules dimerised back-to-back and are closely associated without steric interference. In this model, the C-terminus of the heavy chain can be directly linked to the -13 residue of the light chain variable region with minimum reorganisation of the remaining polypeptide backbone.

Figure 17 shows a schematic representation of non-covalently and covalently joined scFv dimers respectively.

Sequence I.D. 1 shows an example of a mouse Ly-2^{+}Ly-3 V domains construct designed for bacterial expression.

Sequence I.D. 2 shows an example of a human single-chain CD8 construct designed for expression in a bacterial secretion vector such as pPOW.

Sequence I.D. 3 shows an example of a mouse MHC α3 domain designed for expression.

Sequence I.D. 4 shows the DNA sequence of the linkerless 1C3 scFv in pHFA.

Sequence I.D. 5 shows the DNA sequence of the anti influenza NC10 scFv with the pel B secretion signal and the FLAG C-terminal peptide.

Sequence I.D. 6 shows the DNA sequence of the first 1443 bases of the anti-glycophorin 1C3Fab fragment in pHFA ready for ligation post PCR amplification for ligation into p569.

Preferred embodiments of the invention include the following:

1. The structure of the target binding polypeptides may be based on scFv molecules in which one TBR is formed by six surface polypeptide loops to provide contact region to antigen, and hence specificity. In a particularly preferred embodiment, the TBR may be formed by four CDR

loops for contact with antigen to provide sufficient contact area and affinity (Figure 14). Our results using NC10 indicate that it is feasible to randomly mutate these polypeptide sequences to modify target affinity.

2. Bifunctional or polyfunctional reagents can be produced by covalent linkage of individual target binding polypeptides. The covalent linkage can be provided by polypeptide chains in the manner of single-chain Fv molecules. Specificities can be linked by joining together individual proteins, regions which have a propensity for dimerisation or aggregation. Thus it is not necessary for example to link two single-chain Fv fragments in their normal orientation by an additional polypeptide chain, but this can be achieved by linking Heavy and Light chains of differing specificity, or Heavy to Heavy chains, Light to Light chains which can then associate to form functional dimers or aggregates. Of course the method can also be used to join V domains with required specificity to other protein domains, including the Immunoglobulin-like domains derived from CD8, T-cell receptor fragments, or MHC fragments.

In a preferred embodiment the covalent joining of two Ig-like domains, such as the heavy and light antibody variable regions, can be produced with or without a linker polypeptide. For monomeric scFv fragments the polypeptide linker covalently joins the V_H and V_L domains between the carboxyl terminal of one variable (V) domain to the amino terminal of the other V domain without compromising the fidelity of the scFv binding site. The scFv may be assembled in either domain order as a V_H-linker-V_L fusion protein or as a V_L-linker-V_H fusion protein. The linker should preferably be hydrophilic in nature to prevent it from associating with hydrophobic V domain surfaces. The lengths of the linker may be less than 25 amino acid

residues with a preferred size established by empirical selection. A preferred linker sequence consists of pentameric units of Gly4Ser in which the serine residues enhance the hydrophilic characteristics of the peptide backbone, while the glycyl residues give the linker enough flexibility to adopt a range of conformations around the V domains. In a special preferred embodiment the covalent association of polymeric Fv fragments can be produced without an additional linker polypeptide by removal of a segment of one Ig-domain at the junction sequence. These linker-minus constructs are referred to herein as tightly coupled domains (TCDs). The number of amino acids to be removed can be determined either empirically or with the aid of protein design considerations. Figure 16 depicts the association of two Fv molecules "back-to-back" as TCDs and in which the two TBRs are at opposite ends of the molecule thus forming a bifunctional reagent capable of cross-linking two target molecules. In this example, preferably up to 13 amino acids are removed for close association. The resultant molecule has a propensity for oligermerisation, at least to dimers, with a close but not sterically inhibited interaction between the Ig-like domains. It will be appreciated that polypeptide tails can be added at the free amino and carboxyl terminii to increase the number of TBRs on the molecule. It will also be obvious that the missing polypeptide sequences that had been removed at the junction of Ig-like domains can be replaced, in whole or in part, by providing the polypeptide sequences attached to another position in the Ig-like domains. We anticipate that these molecules will be capable of forming two-dimensional arrays thereby providing a bifunctional surface. It will be appreciated that these arrays will have special application as biological coating devices.

3. The complete three-dimensional structure of mature human or mouse CD8, comprising heterodimeric α and β chains, is not yet known. Predictions from a crystal structure of homodimeric human CD8α suggests that the α chains are similar in topology to antibody V_L domains (Leahy *et al*, Cell, 1992 68 1145-1162). We have constructed single-chain variants of mouse CD8α/β heterodimer for expression using bacterial secretion vectors, and similar results would be expected using human CD8. Native human or mouse CD8 molecules are presumed to have affinity only for MHC Class I molecules. We predict that random library approaches, such as those described in the Examples herein, will enable scCD8 molecules to be used as a stable framework for the production of target binding polypeptides. By this process, scCD8 molecules can be used as antibody mimics. Furthermore, the scCD8 molecules can be further modified in the size and conformation of CDR-equivalent loop structure to provide a framework for less than six CDR loops in the contact surface. In a particularly preferred embodiment, we envisage a stable protein framework capable of providing four or five CDR loops in the contact region. We also envisage the strategy to apply to other Ig domains. For example the immunoglobulin-like domains of MHC Class I and II can be expressed in soluble form and when modified can be used as immunomodulatory reagents.

4. Modifications to target binding polypeptides such as those described above can be based on mutation of the coding region, by the use of library selection and modification strategies such as those shown in Figure 3, to mature a single TBP or TBP library of low affinity and wide specificity to enhance the range of target molecules which it recognises, but more importantly to produce a range of

binding affinities for each member of the library, the individual DNA coding regions of which may be easily selected and isolated by modifications of known methodologies. It is envisaged that such a library will comprise antibody-like fragments, or any other peptide which shows an affinity for a ligand or another protein, enzyme or receptor. This may also include a stable core polypeptide which is not in itself antigenic, but may be modified by the addition of CDR loops or peptides with an affinity for specified ligands by grafting the coding regions by recombinant DNA techniques. It can also be seen that a change or changes to the framework regions may result in a change of conformation of the protein such that an altered binding surface is presented, with binding properties different from those of the parent molecule.

The most trivial example includes the construction of expression libraries that produce recombinant antibody fragments (including single-chain Fv fragments) with predetermined target binding specificity. *In vitro* mutation and affinity maturation provide means of presenting the binding molecule such that the appropriate coding regions are selected and retained. Presentation vectors which will allow continual reassortment of the binding domains (which in this example as a preferred embodiment will encompass V_H and V_L domains) subsequent to each of the selection steps shown in Figure 3 can suitably be used, for example pHFA.

5. The invention may be used for the construction and selection of a wide range of receptors, receptor-like molecules and molecules constructed with mutations in potentially critical regions for both binding, structural integrity and biological activity. Initially phage surface presentation after expression and phage rescue from *E. coli* is used to monitor the efficacy of this approach, but other

systems such as the eukaryotic systems are also expression competent. Yeast (*Saccharomyces cerevisiae*) has been shown to express the V_H of NC41, a monoclonal antibody directed against influenza virus neuraminidase, under the control of the alpha mating factor promoter, and the slime mould *Dictyostelium discoideum* is able to express recombinant proteins including both V_H and the scFv of NC10.

6. The specific selection of target-binding polypeptides able to bind to the specified antigens (which may include LIF, TGF-α, glycophorin, cell surface markers or other cell specific surface proteins), is made possible as a result of the presentation on the display vector, for example the presentation on the phage surface of these peptides fused to the Gene III product. Having selected the appropriate phages, they are then subjected to rounds of mutation, as shown in Figure 5.

In the following examples, the *mutD* and *mutT1* mutator strains of *E. coli* are used to induce mutations at random throughout the molecule. This is done by transformation of these *E. coli* strains with the plasmid DNA by any of the standard techniques that appear in the literature; the preferred method is by electroporation. Alternatively the recombinant phage may be transfected into the mutator strains by standard transfection methods. After rounds of growth of these plasmid/phage-bearing *E. coli*, the phage may be rescued by standard techniques with a helper phage, and can then be used in antigen-binding assays to determine the effects of various mutations on the binding affinity.

These mutations are not confined to base substitutions in the DNA, but may also encompass the addition of peptides to the structure of the molecule such that the number, size and location of the binding regions in the molecule is altered. A single domain binding unit

with these additions will show binding characteristics of substantially altered affinity if not specificity. The correlation between mutation at specified sites and the binding affinity may then be used to design novel CDR loops and framework regions for target binding polypeptides with therapeutic and diagnostic potential.

Also included in the scope of the invention is the expression of recombinant proteins from recombinant cells under the direct control of the antigen, or some other ligand which is responsible for the first step in the process towards controlled expression of the "antibody genes".

7. Bifunctional or polyfunctional reagents can be selected using the library technology described above. Target binding polypeptides may be displayed for affinity selection by attachment through a polypeptide tail. Selection based on affinity to two or more different target antigens or haptens will select a single molecule which has two binding surfaces at different positions of the same molecule. The binding surfaces can be overlapping. To construct a library for selection of bifunctional or polyfunctional reagents, the strategy of site specific and random mutagenesis applied to two or more surfaces of the protein molecules may be used. In the case of single-chain Fv or CD8, the preferred regions for mutation will be the CDR loops and their opposite counterpart loops at the other end of the scFv molecule. In the case of Fab molecules, the preferred mutations will be at CDR loops and the opposite counterpart loops at the other end of the constant domains.

Unless otherwise specifically stated, all standard methods referred to herein are to be found in "Molecular Cloning-A Laboratory Manual" Sambrook *et al* 1990.

Example 1 — Construction and Expression of Target Binding Polypeptides as Single Chain Fv Fragments Using Polypeptide Linkers of Different Lengths

A parent scFv fragment of NC10 (a monoclonal antibody that recognises the neuraminidase (NA) molecule on the N9 strain of influenza virus) was designed, constructed and expressed in *E. coli* (Sequence I.D. 5). The amino terminal secretion signal PelB directed the synthesised protein into the *E. coli* periplasm where it became associated with the insoluble membrane fraction. An octapeptide (FLAG; IBI USA) tail was fused to the carboxyl-terminal of the scFv and was used to monitor the scFv through subsequent purification procedures. This reagent is bifunctional with specificity to both neuraminidase and anti-FLAG antibodies.

The scFv NC10 protein was purified by solubilization of the *E. coli* membrane fraction with guanidinium hydrochloride followed by column chromatography. Size exclusion HPLC of purified scFv NC10 showed that the scFv fragment emerged in two peaks corresponding in size to monomers (27 kDa) and dimers (54 kDa). Furthermore, the monomeric form bound to N9 NA to form a complex of ~320 kDa while the dimeric form bound to N9 NA to form a complex of ~640 kDa. The 320 kDa complex could consist of four scFv molecules binding to a single NA molecule, while the 640 kDa complex could consist of four scFv molecules binding to two NA molecules. Electron microscopy confirmed the tight coupling of two neuraminidase tetramers by four bifunctional scFv dimers (Figure 15). High resolution electron microscopy was performed on the tern N9 (avian) strain of influenza neuraminidase complexed with scFv constructs of the NC10 of the Mab, where the molecular complexes were stained

(contrasted) either with potassium phopho-tungstate at pH 7.0 or with uranyl acetate at pH 4.0.

Based on our previous extensive experience of imaging molecular complexes of the same N9NA with monoclonal Fabs (32/3, NC35 and NC41) and with whole monoclonal IgGs (32/3, NC41 and NC10), we were able to interpret the N9Na-scFv complex images as closed structures of pairs of neuraminidase heads coupled together face-to-face by four bridging scFv dimers in such a manner as to maintain four-fold point-group symmetry of this densely packed molecular complex (Figure 15). This image interpretation of the N9Na-scFv molecular complex is directly compatible with the observed molecular weight of the complex in solution of M_r - 610,000.

X-ray diffraction analysis of crystals in which the scFv is complexed with neuraminidase (Figure 14) demonstrates a close association between two scFv molecules related by a two-fold axis of rotation. Two possible dimeric conformations are possible. In the first instance the V_H and V_L domains encoded by a single polypeptide chain with additional peptide tails form a bifunctional scFv which associates non-covalently with the separate scFv molecule (Figure 17). In the second instance, the V_H and V_L domains forming the antigen binding surface (the TBR) in each Fv are non-covalently associated and the two Fvs are covalently joined by the linker polypeptide (Figure 17).

Molecular modelling studies (Figure 14) indicate the distance between the V_H and V_L domains of a non-covalently associated dimer would be at least 35Å, whereas the distance between the V_H and V_L domains of a covalent dimer would be less than 25Å. Given the 3.8Å (0.38nm) distance between adjacent peptide bonds and the distance

lengths that the linkers can theoretically span (54Å, 36Å, 18Å and 0Å for the 15, 10, 5 and 0 residue linker pPOW-scFv NC10 constructs respectively) we examined the type of scFv-NA complexes formed when scFv proteins with different linker lengths bind to antigen.

A series of scFv NC10 proteins with shortened linker lengths were constructed (Figure 5). The first pPOW-scFv NC10 construct has a polypeptide linker consisting of three pentameric Gly4Ser units (this pPOW-scFv NC10 construct was referred to as the 15 residue linker, V_H.15.V_L). The deletion linker mutants were constructed by sequentially removing each of these pentameric units to form constructs with two, one and zero units (referred to as the V_H.10,V_L, V_H.5.V_L and V_H.V_L residue linker pPOW-scFv NC10 constructs respectively). Furthermore, a scFv NC10 construct was made by deleting the first β-strand of the V_L domain (the first 13 amino acids) so that the carboxyl-terminal of the V_H domain joined directly to the V_L domain (V_H.-13.V_L).

<u>Detailed Construction of pPOW-scFv NC10 with Shortened Linker Lengths</u>

The pPOW-scFv NC10 construct was digested successively with *BstE* II (New England Biolabs) and *Sac* I (Pharmacia) according to manufacturers' specifications and the polypeptide linker released. The restricted linkerless pPOW-scFv NC10 DNA was electroeluted from an 0.8% agarose gel and the DNA concentrated by precipitation with 0.3M Na acetate and 2.5 volumes of ethanol. Synthetic oligonucleotides were phosphorylated at their 5' termini by incubating at 37°C for 30 min with 0.5 units of T4 polynucleotide kinase (Pharmacia) and 1mM ATP in One-Phor-All Buffer PLUS (Pharmacia). Pairs of complementary

phosphorylated oligonucleotide primers (Figure 6) were premixed in equimolar ratios to form DNA duplexes encoding single chain linkers of altered lengths. These duplexes were ligated into the *Bst*E II-*Sac* I restricted pPOW-scFv NC10 plasmid using an Amersham ligation kit. A slightly different approach was required to make the V_H.-13.V_L construct. An oligonucleotide primer (Figure 6) spanning the deleted V_L domain was constructed and used in conjunction with a FLAG specific oligonucleotide (Figure 6) to amplify by PCR a V_H.-13.V_L fragment of the scFv NC10. The amplification product was digested with *Bst*E II and *Eco*R I and ligated into similarly digested pPOW-scFv NC10 plasmid using an Amersham ligation kit. The ligation mixtures were purified by extraction with an equal volume of phenol/chloroform and precipitated with 0.3 M Na acetate and 2.5 volumes of ethanol. The ligated DNA was resuspended in 20 ml H20 and 5 ml of the sample was transformed into *E. coli* DH5a (*sup*E44, *hsd*R17, *rec*A1, *end*A1, *gyr*A96. *thi*-1, *rel*A1) and LE392 (*sup*E44, *sup*F58, *hsd*R14, *lac*Y1, *gal*K2, *gal*T22, *met*B1, *trp*R55). Cells were shaken in 1 ml of LB medium for 1 hr and plated onto 2xYT medium with 100 mg/ml ampicillin. Recombinant clones were identified by PCR screening with oligonucleotides directed to the PelB leader and FLAG sequences of the pPOW vector. The DNA sequence of the shortened linker regions were verified by sequencing double-stranded DNA using Sequenase 2.0 (United States Biochemical).

Protein Expression of the scFv NC10 Proteins with Shortened Linkers

Transformed LE392 were grown overnight at 30°C in SB medium and diluted 1:10 to inoculate fresh SB medium. Cultures were grown at 30°C with shaking until the absorbance at 600nm (A_{600}) was approximately four. The

temperature was raised to 42°C for the remainder of the induction period (which continued for 4 hr until the A_{600} ~7). Cells were recovered by centrifugation (Beckman JA10 6,000 rpm for 15 min) and the supernatant fraction removed. The cell pellet was resuspended in 10% of the original volume in 20% sucrose, 10 mM Tris.HCl. pH7.5 and left on ice for 5 min. EDTA was added to a final volume of 5 mM and the mixture incubated on ice for a further 10 min and centrifuged as before to pellet the cells. The supernatant was discarded and the cell pellet resuspended in H_2O, the mixture was recentrifuged and the supernatant containing the periplasmic proteins removed. The resulting cell pellet was resuspended in H_2O and lysed by sonication (six 30 sec bursts for large scale preparations and one 30 sec burst for small scale preparations) and kept on ice for 5 min. After centrifugation the aqueous phase was recovered as the solubilized cytoplasmic fraction while the pellet contained the insoluble membrane-associated fraction. To verify scFv NC10 expression total cell lysate from individual clones were analysed by SDS-PAGE under reducing conditions and Western blotting using the anti-FLAG monoclonal antibody, M2 (Figure 7). Single positive bands migrating at ~28, 29, 31 and 32 kDa were observed (Figure 7, lanes 13-16) which correlate with the anticipated Mr of the scFv NC10-FLAG fusion protein synthesised by pPOW-scFv NC10 constructs with 0, 5, 10 and 15 residue linkers respectively. ScFv NC10 proteins with 0, 5, and 10 residue linkers showed the same characteristics as the 15 residue linker. The scFv NC10-FLAG fusion proteins were associated with the insoluble membrane fraction of *E. coli*, approximately half of which could be solubilized by treating with guanidinium hydrochloride.

The soluble products were purified by gel filtration and chromatography on Mono-Q or on an affinity

matrix containing an antibody specific for the tail moiety. The pure products were characterized by SDS-PAGE, size exclusion HPLC (SE-HPLC), ultracentrifugal analysis, binding activity towards the parent antigen (influenza virus neuraminidase), electron microscopy of the complexes formed between the antigen and reagent. Cross linking experiments confirmed the size of the products. The properties are summarized in Figure 8.

<u>Example 2</u> <u>Construction of mouse and human scCD8</u>

The α and β chains containing only the V-like domains of mouse CD8 were amplified separately by PCR with Vent polymerase using primers containing homology to the V-like domains (using available database sequences) and with additional nucleotides encoding the $(Gly_4Ser)_3$ linker (Sequence I.D. 1). After annealing the two separate domains the products were extended using dNTPs and polymerase. The scCD8 gene was amplified using new primers containing *Msc*I site at the 3' end and *Sal*I site at the 5' end. The single chain product was digested with *Msc*I and *Sal*I then cloned into *Msc*I and *Sal*I digested pPOW vector. High level protein synthesis was obtained in *E. coli* host cell strain pop2136. An N-terminal modification was designed to increase the synthesis of correctly cleaved product which was achieved by changing the N-terminal residue of mouse CD8 α chain from Lysine to Serine (the Human CD8 a chain N-terminal residue is a Serine). The synthesis of scCD8 in pPOW using *E. coli* host cell strain pop2136, showing whole cell lysates, can be seen in Figure 10. The synthesised scCD8 product was detected by anti-CD8 antibodies that only recognise protein in the conformationaly correct form.

Human scCD8

The DNA encoding the V-like domain of the mature α chain protein was amplified by PCR using Taq polymerase and primers containing homology to the V-like domain (using available database sequences) with additional nucleotides encoding the $(Gly_4Ser)_3$ linker and incorporating restriction enzyme sites *Msc*I and *Bam*HI (Sequence I.D. 2). The V-like domain of the CD8 β chain was amplified by PCR directly from DNA isolated from blood using primers containing *Bam*HI and *Eco*RI restriction enzyme sites. The two individual products were digested with the appropriate enzymes then ligated into *Msc*I and *Eco*RI digested pPOW vector.

The DNA sequence of each of the single chain CD8 constructs was confirmed by double stranded DNA sequencing. The nucleotide sequence can be seen in Sequence I.D. 1 and 2. In this example the vector directs the synthesis of a scCD8 with a C-terminal peptide tail for diagnostic and coupling applications, including affinity purification.

Preferred techniques to monitor the biological activity of the scCD8 product include:

a) Direct measurement of protein binding affinity for example using biosensor technology or ultracentrifugation using binding to whole cells, cell surface molecules or their fragments such as β2 microglobulin or the α3 domains of the MHC class I molecule.

b) measurement of binding to the MHC class I molecules expressed in RMA-S cells (peptide loaded) using the C-terminal peptide tails as diagnostic markers.

c) an interference of function assay such as monitoring changes to the peptide induced

dose-dependent effect on IL2 production during T-cell activation.

Example 3 Construction of Linkerless AntiGlycophorin 1C3

The parent IC3 antibody and scFv derivatives are disclosed in the International Patent Application No. PCT/AU93/00228.

Oligonucleotide N2034 (5'-ACGTAGGTCACCGTCGCCTCCGACATCGTCATGTCACAGTCTCCATCCTCC-3') was synthesised to have complementarity to the last 15 bases at the 3' end of 1C3 V_H coupled directly to the first 30 bases of the 1C3 V_L 5' sequence without any intervening linker sequence.

Oligonucleotide N2035 (5'-TTTATAATCTGCGGCCGCCCGATTAATTTC-3') was synthesised to have complementarity to the 1C3 V_L sequence on the opposite strand around the Not I site near the 3' end.

The two oligonucleotides were used with 1C3 template DNA in a Polymerase Chain Reaction to produce a 1C3 product of 3'-V_H sequence juxtaposed to 5'-V_L sequence flanked by Bst EII and Not I restriction endonuclease sites.

After incubation of the PCR product with restriction endonucleases Bst EII and Not I, the resultant fragment was ligated with vector pHFA containing the 1C3 scFv sequence previously digested with Bst EII and Not I to remove the intervening sequence. The ligated product was used to transform *E. coli* strain TG1. Transformant colonies containing inserts were verified as containing the DNA sequence as shown in Sequence I.D. 4.

This gene construct was expressed in this vector and related vectors when transferred to a non-suppressor

E. coli strain and induced with IPTG, or by transferring the 1C3 coding region to the thermoinducible expression vector pPOW.

<u>Example 4</u> <u>Mutation with mutator strains of *E. coli*</u>

The NC10 scFv plasmid coding for the expression of the recombinant antineuraminidase antibody NC10 scFv was electroporated into *E. coli mutD*. Mutants were produced by subjecting the samples to the mutation cycle shown in Figure 11. They were grown for 50 generations in exponential phase (to induce mutation of the phasmid DNA) in YT+AMP+TET and then rescued with the helper phage. The rescued phage was applied to the immunotubes previously coated with 10μg/ml of the antigen, non-binding phage removed by washing with PBS etc and the specifically bound phage eluted with 100mM triethylamine, collected into 0.5 volumes of 1M Tris-Hydroxymethylmethylamine-HCl pH7.5 and then transfected into *mutD* cells by standard methods, (unless otherwise specfically stated all standard methods referred to herein are to be found in "Molecular Cloning-A Laboratory Manual" Sambrook *et al*, 1990) and again grown through 50 generations whilst maintaining the cells in the logarithmic phase of growth. After an appropriate number of rounds of mutation selection which in this example is three the phage titres are in the region of 10^7-10^8 phage/ml. After the final panning step, eluted phage were transfected into *E. coli* TG1 cells and plated onto YT+AMP+Glucose plates and then each of the isolated colonies grown before phage rescue and analysis by ELISA on "flu" virus or glycophorin. The colonies which exhibited non-wild-type levels of ELISA activity, were then amplified, the DNA sequenced and the phage transfected into *E. coli* HB2151 cells available from the American Type Culture Collection, for soluble expression. The phage were

transfected into HB2151 by the standard methods and the selected individual colonies of each phage sample grown in YT+AMP(100μg/ml) prior to induction with 1mM IPTG (isopropylthiogalactoside) for 4 to 16h at 37°C, with or without subsequent incubation at 4°C for 16h. The culture supernatant and the extracts of periplasm, cell membranes and cell cytoplasm were collected and analysed for the recombinant gene expression as described (Power *et al*, 1992, Gene)

Example 5

The recombinant 1C3 scFv (a glycophorin-binding antibody coding region) in the phagmid pHFA prepared as described in International Patent Application No. PCT/AU93/00228 was subjected to random mutation in the *mutD E. coli* as discussed in Example 4, and the selection protocol similarly applied, with the exceptions that the selection involved coating the solid phase matrix (ELISA plate, Immunotube, or latex bead) with glycophorin A from a 10μg/ml solution in PBS. The results of the ELISA screening for selection of individual colonies is illustrated in Figure 12. Competitive ELISA assays, using detection with anti-FLAG antibody, were performed on selected colonies after mutation, and Table 1 shows the increases in relative affinity of the expressed proteins for the antigen asialoglycophorin.

Table 1

recombinant cloned scFv	Mutation	Affinity nM (off rate)
1C3	wt	62
1C3.A13		40
1C3.B7		29

Example 6

A scFv library in the phagemid vector pHEN (MedicalResearch Council, U.K.) was transferred into the *mutD* strains of *E. coli* and treated as for Examples 4 or 5 for the mutation, detection and selection of scFv with modified binding ability. Selecting for glycophorin binders. To increase the range of glycophorin-binding antibodies available the naive scFv library was used as the starting point for this maturation and affinity selection of phage displayed scFvs. Two of the unique anti-glycophorin scFvs that were selected from the naive scFv phage display library, have the deduced amino acid sequence shown for the region of their Vk4 chains that were subsequently shown to be mutated are shown in Table 2.

Table 2

FTASTGDVPDRFSGSGSGTDFTLRISSLQAEDVAVYYCQQASVFP
CIYWNPDSPDRFSGSGSGTDFTLRISLQAEDVAVYYCQQASVFP

Affinity maturation of each of these molecules was achieved by using the mutation (*mut* D5) affinity selection cycle, as we show in Figure 11, and the changes that result to a subset of the mutated molecules is shown in Table 3.

Table 3

scFv selected from Naive Library	Mutation A.A (position)	Affinity μM (off rate)
A9	wt	48
A9.5	S-V (98)	15
A9.13	G-D (63)	24
	V-S (64)	0.06
E3	wt	18
E3.1	S-Q (82)	9
E3.2	S-T (83)	2
C12	wt	11
C12.1	G-Q (48)	35
C12.2	L-G (15)	1

Example 7

Expression of the antibody-fragment coding regions in *D. discoideum* is from the vector pAV1 which has has been constructed from the parent vector p569 (a gift from W. Nellen, Max-Planck Institute, Munich, Germany) and the V_H coding region of the NC41V_H as described below. The vector p569 is shown in Figure 13; this is one of a family of vectors that are *E. coli/D. discoideum* shuttle vectors using the α-L fucosidase promoter and signal sequence for the secretion of the expressed "ligand binding peptide" to the cell surface. Table 4 shows the results from the immunodot-blot of the expression of the Influenza NC41 V_HFLAG detected by the antiFLAG antibody (M2).

The V_H coding sequence of the monoclonal antibody NC41 was amplified by Polymerase Chain Reaction using the oligonucleotide sequences:

N849

5' CCTTGCCTGCAGGTCGACCTATGGACAGGTGCAGCTGCAGCAG 3'

N863

5' TTACCATGGTTACTTGACCTTAATCAGCAGGACAAATGAAATAAATTTATCATCAT CATCTTTATAATC 5'

N849 contains sequence complementary to the N-terminus of the $NC41V_H$ coding region together with the α-L-fucosidase signal sequence and cleavage site, as well as SalI restriction site suitable for cloning into the expression vector p569.

N863 contains sequence complementary to the FLAG coding sequence of the $NC41V_H$, together with a transmembrane hydrophobic sequence, an NcoI restriction site for cloning and a translation stop codon.

DNA of the vector pAV 569 (a gift from W. Nellen, Max-Planck Institute, Martinsreid, Germany) was digested with the restriction enzyme *Nco*I and *SaiI*, and the cut vector was purified by the standard techniques of agarose electrophoresis and phenol extraction.

The PCR amplified and restriction digested $NC41V_H$ FLAG was ligated into the vector and the mixture was transformed into *E. coli*. Recombinant colonies were selected on ampicillin-containing YT plates and recombinant plasmids were recovered, purified and identified using standard techniques. The recombinant plasmid is designated pAV1.

The recombinant plasmid pAV1 was transformed into vegetative cells of *D. discoideum* by the feeding method disclosed in GB-2159821, by Friendlender and Mella.

Recombinant *D. discoideum* were selected using the antibiotic G418 at 10μg/ml on DMB medium. Recombinant *D. discoideum* amoebae were grown in 2ml cultures of DMB medium containing 10μg/ml G418. After growth for 48 hours

at 22°C, dot blot analysis was performed on 100μl aliquots of the culture supernatant, and an anti-FLAG antibody was used to detect the presence of the NC10V_H FLAG antibody fragment in the culture. The results are summarised in Table 4.

Table 4

EXPRESSION OF NC41V_H-FLAG IN *D. DISCOIDEUM*
Immunodetection Dot Blot with Anti-FLAG Antibody

D. discoideum clone #		Detection by Antibody
Negative Control		-
Negative Control		-
Positive Control		+++
9C	Recombinant *D. discoideum*	+
9D	"	-
9E	"	++
9F	"	-
9G	"	++
8B	"	+++
8C	"	-
8D	"	+++
8E	"	+++
8F	"	-
8G	"	+++
7D	"	+
7E	"	-
7F	"	+
7G	"	-

- = no reaction
+++ = strong reaction
\+ = weak reaction

Example 8

Construction of the recombinant vector containing the 1C3Fab for expression in *D. discoideum*. The structure of the parent vector is shown in Figure 13 (p569),and was modified by removal of a *Bam*HI/*Bgl*II fragment by restriction digestion and religation, leaving unique *Xba*I and *Ssp*I sites for the cloning of the antibody coding regions that were constructed by the polymerase chain reaction with the primers:

5' CAGGTCGACTCTAGAGTATGGGAGGTGAGGCTTCTCGAG 3'

5' AAATTTATAATTATTTATCATCATCATCTTTATAATC 3'

and the 1C3Fab coding region (see Sequence I.D. 6) as template. This Fab is a polyfunctional polypeptide as it combines binding activities (TBRs) for glycophorin; and antiFLAG and anti EEF antibodies. Restriction digestion of the PCR products was followed by standard purification, ligation and transformation protocols for construction in *E. coli*. Transformation of *D. discoideum* is effected by feeding transformed *E. coli* (see GB-2159821A) or by standard methods by those skilled in the art as published in the literature such as by calcium phosphate crystals (Nellen *et al*, Mol. Cell. Biol., 1984 4 2890-2898) or electroporation (Howard *et al*, 1988, 16 2613-2623) with selection on G418 (geneticin).

It will be clearly understood that the invention in its general aspects is not limited to the specific details referred to hereinabove.

```
File:          B:\POWLY23V.SEQ
Description:   JLApelB Ly-2+Ly-3 V domains linked Msc1-Sal1 with FLAG tail
From base:     1
To base:       849
Total bases:   849

      M  K  Y    L  L  P  T    A  A  A    G  L  L    L  L  A  A    Q  P  A
  1   ATGAAATACC TATTGCCTAC GGCAGCCGCT GGATTGTTAT TACTCGCTGC CCAACCAGCG

      M  A  K    P  Q  A  P    E  L  R    I  F  P    K  K  M  D    A  E  L
 61   ATGGCCAAGC CACAGGCACC CGAACTCCGA ATCTTTCCAA AGAAAATGGA CGCCGAACTT

      G  Q  K    V  D  L  V    C  E  V    L  G  S    V  S  Q  G    C  S  W
121   GGTCAGAAGG TGGACCTGGT ATGTGAAGTG TTGGGGTCCG TTTCGCAAGG ATGCTCTTGG

      L  F  Q    N  S  S  S    K  L  P    Q  P  T    F  V  V  Y    M  A  S
181   CTCTTCCAGA ACTCCAGCTC CAAACTCCCC CAGCCCACCT TCGTTGTCTA TATGGCTTCA

      S  H  N    K  I  T  W    D  E  K    L  N  S    S  K  L  F    S  A  M
241   TCCCACAACA AGATAACGTG GGACGAGAAG CTGAATTCGT CGAAACTGTT TTCTGCCATG

      R  D  T    N  N  K  Y    V  L  T    L  N  K    F  S  K  E    N  E  G
301   AGGGACACGA ATAATAAGTA CGTTCTCACC CTGAACAAGT TCAGCAAGGA AAACGAAGGC

      Y  Y  F    C  S  V  I    S  N  S    V  M  Y    F  S  S  V    V  P  V
361   TACTATTTCT GCTCAGTCAT CAGCAACTCG GTGATGTACT TCAGTTCTGT CGTGCCAGTC

      L  Q  G    G  G  G  S    G  G  G    G  S  G    G  G  G  S    L  I  Q
421   CTTCAGGGTG GCGGAGGCTC AGGCGGTGGT GGATCAGGTG GCGGCGGATC TCTCATTCAG

      T  P  S    S  L  L  V    Q  T  N    H  T  A    K  M  S  C    E  V  K
481   ACCCCTTCGT CCCTGCTGGT TCAAACCAAC CATACGGCAA AGATGTCCTG TGAGGTTAAA

      S  I  S    K  L  T  S    I  Y  W    L  R  E    R  Q  D  P    K  D  K
541   AGCATCTCTA AGTTAACAAG CATCTACTGG CTGCGGGAGC GCCAGGACCC CAAGGACAAG

      Y  F  E    F  L  A  S    W  S  S    S  K  G    V  L  Y  G    E  S  V
601   TACTTTGAGT TCCTGGCCTC CTGGAGTTCT TCCAAAGGAG TTTTGTATGG TGAAAGTGTG

      D  K  K    R  N  I  I    L  E  S    S  D  S    R  R  P  F    L  S  I
661   GACAAGAAAA GAAATATAAT TCTTGAGTCT TCAGACTCAA GACGGCCCTT TCTCAGTATC

      M  N  V    K  P  E  D    S  D  F    Y  F  C    A  T  V  G    S  P  K
721   ATGAATGTGA AGCCAGAGGA CAGTGACTTC TACTTCTGCG CGACGGTTGG GAGCCCCAAG

      M  V  F    G  T  G  T    K  L  T    V  V  D    Y  K  D  D    D  D  K
781   ATGGTCTTTG GGACAGGGAC GAAGCTGACT GTGGTTGATT ACAAGGACGA CGATGACAAG

      *  S  T
841   TAGTCGACA
```

SEQUENCE I.D. 1

```
File:          B:\CD8ACD8B.SEQ
Description:   Human single chain CD8 in pPOW (pelB CD8a and CD8b V dom )
From base:     1
To base:       822
Total bases:   822

      M  K  Y   L  L  P  T   A  A  A   G  L  L   L  L  A  A   Q  P  A
  1   ATGAAATACC TATTGCCTAC GGCAGCCGCT GGATTGTTAT TACTCGCTGC CCAACCAGCG

      M  A  S   Q  F  R  V   S  P  L   D  R  T   W  N  L  G   E  T  V
 61   ATGGCCAGCC AGTTCCGGGT GTCGCCGCTG GATCGGACCT GGAACCTGGG CGAGACAGTG

      E  L  K   C  Q  V  L   L  S  N   P  T  S   G  C  S  W   L  F  Q
121   GAGCTGAAGT GCCAGGTGCT GCTGTCCAAC CCGACGTCGG GCTGCTCGTG GCTCTTCCAG

      P  R  G   A  A  A  S   P  T  F   L  L  Y   L  S  Q  N   K  P  K
181   CCGCGCGGCG CCGCCGCCAG TCCCACCTTC CTCCTATACC TCTCCCAAAA CAAGCCCAAG

      A  A  E   G  L  D  T   Q  R  F   S  G  K   R  L  G  D   T  F  V
241   GCGGCCGAGG GGCTGGACAC CCAGCGGTTC TCGGGCAAGA GGTTGGGGGA CACCTTCGTC

      L  T  L   S  D  F  R   R  E  N   E  G  Y   Y  F  C  S   A  L  S
301   CTCACCCTGA GCGACTTCCG CCGAGAGAAC GAGGGCTACT ATTTCTGCTC GGCCCTGAGC

      N  S  I   M  Y  F  S   H  F  V   P  V  F   L  P  A  G   G  R  G
361   AACTCCATCA TGTACTTCAG CCACTTCGTG CCGGTCTTCC TGCCAGCGGG CGGCCGCGGT

      S  G  G   G  G  S  G   G  G  G   S  L  Q   Q  T  P  A   Y  I  K
421   TCAGGTGGAG GTGGATCCGG AGGCGGTGGA TCTCTCCAGC AGACCCCTGC ATACATAAAG

      V  Q  T   N  K  M  V   M  L  S   C  E  A   K  I  S  L   S  N  M
481   GTGCAAACCA ACAAGATGGT GATGCTGTCC TGCGAGGCTA AAATCTCCCT CAGTAACATG

      R  I  Y   W  L  R  Q   R  Q  A   P  S  S   D  S  H  H   E  F  L
541   CGCATCTACT GGCTGAGACA GCGCCAGGCA CCGAGCAGTG ACAGTCACCA CGAGTTCCTG

      A  L  W   D  S  A  K   G  T  I   H  G  E   E  V  E  Q   E  K  I
601   GCCCTCTGGG ATTCCGCAAA AGGGACTATC CACGGTGAAG AGGTGGAACA GGAGAAGATA

      A  V  F   R  D  A  S   R  F  I   L  N  L   T  S  V  K   P  E  D
661   GCTGTGTTTC GGGATGCAAG CCGGTTCATT CTCAATCTCA CAAGCGTGAA GCCGGAAGAC

      S  G  I   Y  F  C  M   I  V  G   S  P  E   L  T  F  G   K  G  T
721   AGTGGCATCT ACTTCTGCAT GATCGTCGGG AGCCCCGAGC TGACCTTCGG GAAGGGAACT

      Q  L  S   V  V  D  Y   K  D  D   D  D  K   *
781   CAGCTGAGTG TGGTTGATTA CAAGGACGAC GATGACAAGT AG
```

SEQUENCE I.D. 2

```
File:          B:\MHCA3.SEQ
Description:   MHCI a3 H-2K domain in pPOW pelB Msc-EcoRI (no FLAG)
From base:     1
To base:       371
Total bases:   371

      M  K  Y   L  L  P  T   A  A  A   G  L  L   L  L  A  A   Q  P  A
  1   ATGAAATACC TATTGCCTAC GGCAGCCGCT GGATTGTTAT TACTCGCTGC CCAACCAGCG

      M  A  K   A  H  V  T   H  H  R   R  P  E   G  D  V  T   L  R  C
 61   ATGGCCAAGG CCCATGTCAC CCATCACCGC AGACCTGAAG GTGATGTCAC CCTGAGGTGC

      W  A  L   G  F  Y  P   A  D  I   T  L  T   W  Q  L  N   G  D  E
121   TGGGCCCTGG GCTTCTACCC TGCTGACATC ACCCTGACCT GGCAGTTGAA TGGGGACGAG

      L  T  Q   E  M  E  L   V  E  T   R  P  A   G  D  G  T   F  Q  K
181   CTGACCCAGG AAATGGAGCT TGTGGAGACC AGGCCTGCAG GGGATGGAAC CTTCCAGAAG

      W  A  S   V  V  V  P   L  G  K   E  Q  K   Y  T  C  H   V  E  H
241   TGGGCATCTG TGGTGGTGCC TCTTGGGAAG GAGCAGAAGT ACACATGCCA TGTGGAACAT

      E  G  L   P  E  P  L   T  L  R   W  G  K   E  E  P  P   S  S  T
301   GAGGGGCTGC CTGAGCCCCT CACCCTGAGA TGGGGCAAGG AGGAGCCTCC TTCATCCACC

      K  *  N
361   AAGTAGAATT C
```

SEQUENCE I.D. 3

Linkerless 1C3 as Constructed in pHFA.
From Hind III site in pHFA to start of gene 3 sequence.

```
                                                         M   K   Y
  1 aag ctt gca tgc aaa ttc tat ttc aag gag aca gtc ata ATG AAA TAC

     L   L   P   T   A   A   A   G   L   L   L   L   A   A   Q   P
 49 CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC GCG GCC CAG CCG

     A   M   A   E   V   K   L   Q   E   S   G   G   G   P   V   Q
 97 GCC ATG GCC GAG GTG AAG CTG CAG GAG TCT GGA GGT GGC CCG GTA CAA

     P   G   G   S   L   K   L   S   C   A   A   S   G   F   D   F
145 CCT GGA GGA TCC CTG AAA CTC TCC TGT GCA GCC TCA GGA TTC GAT TTT

     S   R   Y   W   M   N   W   V   R   R   A   P   G   K   G   L
193 AGT AGA TAC TGG ATG AAT TGG GTC CGG CGG GCT CCA GGG AAG GGG CTA

     E   W   I   G   E   I   N   Q   Q   S   S   T   I   N   Y   S
241 GAG TGG ATT GGA GAA ATT AAT CAA CAA AGC AGT ACG ATA AAC TAT TCG

     P   P   L   K   D   K   F   I   I   S   R   D   N   A   K   S
289 CCA CCT CTG AAG GAT AAA TTC ATC ATC TCC AGA GAC AAC GCC AAA AGT

     T   L   Y   L   Q   M   N   K   V   R   S   E   D   T   A   L
337 ACG CTG TAC CTG CAA ATG AAC AAA GTG AGA TCT GAG GAC ACA GCC CTT

     Y   Y   C   A   R   L   S   L   T   A   A   G   F   A   Y   W
385 TAT TAT TGT GCA AGA CTT TCT CTT ACT GCG GCA GGG TTT GCT TAC TGG

     G   Q   G   T   L   V   T   V   A   S   D   I   V   M   S   Q
433 GGC CAA GGG ACT CTG GTC ACC GTC GCC TCC GAC ATC GTC ATG TCA CAG

     S   P   S   S   L   A   V   S   V   G   E   K   V   T   M   S
481 TCT CCA TCC TCC CTG GCT GTG TCA GTA GGA GAG AAG GTC ACT ATG AGC

     C   R   S   S   Q   S   L   F   N   S   R   T   R   K   N   Y
529 TGC AGA TCC AGT CAG AGT CTG TTC AAC AGT AGA ACC CGA AAG AAC TAC

     L   T   W   Y   Q   Q   K   P   G   Q   S   P   K   P   L   I
577 TTG ACT TGG TAC CAG CAG AAA CCA GGG CAG TCT CCT AAA CCG CTG ATC

     Y   W   A   S   T   R   E   S   G   V   P   D   R   F   T   G
625 TAC TGG GCA TCC ACT AGG GAA TCT GGG GTC CCT GAT CGC TTC ACA GGC

     S   G   S   G   T   D   F   T   L   T   I   S   S   V   Q   A
673 AGT GGA TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AGT GTG CAG GCT

     E   D   L   A   D   Y   Y   C   K   Q   S   Y   N   L   R   T
721 GAA GAC CTG GCA GAT TAT TAC TGC AAG CAA TCT TAT AAT CTT CGG ACG

     F   G   G   G   T   K   L   E   I   N   R   A   A   A   D   Y
769 TTC GGT GGA GGC ACC AAG CTG GAA ATT AAT CGG GCG GCC GCA GAT TAT

     K   D   D   D   D   K   *   A   A   *   T   V   E   S   C   L
817 AAA GAT GAT GAT GAT AAA TAG GCC GCA TAG ACT GTT GAA AGT TGT TTA

     A   K
865 GCA AAA........
```

SEQUENCE I.D. 4

```
                    pelB signal sequence
M K Y L L P T A A A G L L L L A A Q P A
ATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCTGCCCAACCAGCG

   H1   Pst1        H10
M A Q V Q L Q Q S G A E L V K P G A S V
ATGGCGCAGGTGCAGCTGCAGCAGTCTGGGGCTGAACTGGTGAAGCCTGGGGCCTCAGTG

H20               ┌─────H30─────┐
R M S C K A S │G Y T F T N Y│ N M Y W V K
AGGATGTCCTGCAAGGCTTCTGGCTACACATTTACCAATTACAACATGTACTGGTAAAA

H40                        H50      ┌H52A───┐
Q S P G Q G L E W I G I F Y │P G N G│ D T
CAGTCACCTGGACAGGGCCTGGAGTGGATTGGAATTTTTTATCCAGGAAATGGTGATACT

  H60                        H70
S Y N Q K F K D K A T L T A D K S S N T
TCCTACAATCAGAAGTTCAAAGACAAGGCCACATTGACTGCTGACAAATCCTCCAACACA

  H80     H82A  H82C                H90
A Y M Q L S S L T S E D S A V Y Y C A R
GCCTACATGCAGCTCAGCAGCCTGACATCTGAGGACTCTGCGGTCTATTACTGTGCAAGA

  ┌──────────H100──────────H100E┐                BstE2
S │G G S Y R Y D G G F D│ Y W G Q G T T V
TCGGGGGGCTCCTATAGATACGACGGAGGCTTTGACTACTGGGGCCAAGGGACCACGGTC

H110                 linker                  L1
T V S G G G G S G G G G S G G G G S D I
ACCGTCTCCGGTGGTGGTGGTTCGGGTGGTGGTGGTTCGGGTGGTGGTGGTTCGGATATC

Sac1             L10                        L20
E L T Q T T S S L S A S L G D R V T I S
GAGCTCACACAGACTACATCCTCCCTGTCTGCCTCTCTGGGAGACAGAGTCACCATCAGT

        ┌─────────L30───┐                L40
C R A │S Q D I S N Y│ L N W Y Q Q N P D G
TGCAGGGCAAGTCAGGACATTAGTAATTATTTAAACTGGTATCAACAGAATCCAGATGGA

                 ┌L50──┐                 L60
T V K L L I Y │Y T S│ N L H S E V P S R F
ACTGTTAAACTCCTGATCTACTACACATCAAATTTACACTCAGAAGTCCCATCACGGTTC

                 L70                     L80
S G S G S G T D Y S L T I S N L E Q E D
AGTGGCAGTGGGTCTGGAACAGATTATTCTCTCACCATTAGCAACCTGGAACAAGAAGAT

                 L90 ┌──────────┐          L100
I A T Y F C Q Q │D F T L P F│ T F G G G T
ATTGCCACTTACTTTTGCCAACAGGATTTTACGCTTCCGTTCACGTTCGGAGGGGGGACC

   Xho1          FLAG               EcoR1
K L E I R D Y K D D D D K * *
AAGCTCGAGATAAGAGACTACAAAGACGATGACGATAAATAATAAGAATTC
```

SEQUENCE I.D. 5

```
File:          A:\1C3FAB.SEQ
Description:   anti-glycophorin 1C3 Fab
From base:     1
To base:       1443
Total bases:   1443

   1  aaaaaagcGG CCCAGCCGGC CATGGCCGAG GTGAGGCTTC TCGAGTCTGG AGGTGGCCCG
  61  GTACAACCTG GAGGATCCCT GAAACTCTCC TGTGCAGCCT CAGGATTCGA TTTTAGTAGA
 121  TACTGGATGA ATTGGgtcCG GCGGGCTCCA GGGAAGGGGC TAGAGTGGAT TGGAGAAATT
 181  AATCAACAAA GCAGTACGAT AAACTATTCG CCACCTCTGA AGGATAAATT CATCATCTCC
 241  AGAGACAACG CCAAAAGTAC GCTGTACCTG CAAATGAACA AAGTGAGATC TGAGGACACA
 301  GCCCTTTATT ATTGTGCAAG ACTTTCTCTT ACTGCGGCAG GGTTTGCTTA CTGGGGCCAA
 361  GGGACTCTGG TCACTGTCTC TGCAGCCAAA ACGACACCCC CATCTGTCTA TCCACTGGCC
 421  CCTGGATCTG CTGCCCAAAC TAACTCCATG GTGACCCTGG GATGCCTGGT CAAGGGCTAT
 481  TTCCCTGAGC CAGTGACAGT GACCTGGAAC TCTGGATCCC TGTCCAGCGG TGTGCACACC
 541  TTCCCAGCTG TCCTGCAGTC TGACCTCTAC ACTCTGAGCA GCTCAGTGAC TGTCCCCTCC
 601  AGCACCTGGC CCAGCGAGAC CGTCACCTGC AACGTTGCCC ACCCGGCCAG CAGCACCAAG
 661  GTGGACAAGA AAATTgaaga attttaatta aaacatggaa ataaaGTGAA ACAAAGCACT
 721  ATTGCACTGG CACTCTTACC GTTACTGTTT ACCCCGGTAA CCAAAGCCGA CATCGTCATG
 781  TCACAGTCTC CATCCTCCCT GGCTGTGTCA GTAGGAGAGA AGGTCACTAT GAGCTGCAGA
 841  TCCAGTCAGA GTCTGTTCAA CAGTAGAACC CGAAAGAACT ACTTGACTTG GTACCAGCAG
 901  AAACCAGGGC AGTCTCCTAA ACCGCTGATC TACTGGGCAT CCACTAGGGA ATCTGGGGTC
 961  CCTGATCGCT TCACAGGCAG TGGATCTGGG ACAGATTTCA CTCTCACCAT CAGCAGTGTG
1021  CAGGCTGAAG ACCTGGCAGA TTATTACTGC AAGCAATCTT ATAATCTTCG GACGTTCGGT
1081  GGAGGCACCA AGCTGGAAAT TAAACGGGCT GATGCTGCAG TATCCATCTT cCCACCATCC
1141  AGTGAGCAGT TAACATCTGG ATCTGGAGGT GCCTCAGTCG TGTGCTTCTT GAACAACTTC
1201  TACCCCAAAG ACATCAATGT CAAGTGGAAG ATTGATGGCA GTGAACGACA AAATGGCGTC
1261  CTGAACAGTT GGACTGATCA GGACAGCAAA GACAGCACCT ACAGCATGAG CAGCACCCTC
1321  ACGTTGACCA AGGACGAGTA TGAACGACAT AACAGCTATA CCTGTGAGGC CACTCACAAG
1381  ACATCAACTT CACCCATTGT CAAGAGCTTC AACAGGggaG AGTGTgcggc cgcagattat
1441  aaa
```

SEQUENCE I.D. 6

CLAIMS

1. A target-binding polypeptide having:

a) a stable core polypeptide region (SCR); and

b) at least one target-binding region (TBR),

in which the target-binding region(s) are covalently attached to the SCR and which have optionally been subjected to a maturation step in order to modify the specificity, the affinity or the avidity of binding to the target.

2. A polypeptide according to Claims 1 which can self associate to form stable dimers, aggregates or arrays.

3. A polypeptide according to Claim 1 or Claim 2 in which the SCR and the TBR are joined by a linker moiety.

4. A polypeptide according to any one of Claims 1 to 3 which is bifunctional or polyfunctional.

5. A polypeptide according to any one of Claims 1 to 4 in which the SCR is of human origin, and is a protein of a type present in serum or displayed on cell surfaces.

6. A polypeptide according to any one of Claims 1 to 5 in which the TBR is capable of binding to a target selected from the group consisting of glycophorin, or other red blood cell surface proteins, influenza virus neuraminidase, viral antigens, antibodies or other members of the Ig superfamily, transforming growth factor-α (TGF-α), tumour markers, cell surface proteins, and leukaemia inhibitory factor (LIF).

7. A target binding polypeptide according to any one of Claims 1 to 6 with homology to the immunoglobulin superfamily, including modified antibodies or antibody fragments, scFv fragments, modified CD8 molecules, and combinations of antibody molecules or fragments thereof with CD8.

8. A DNA construct encoding a target binding polypeptide according to any one of Claims 1 to 7.

9. A method for producing a DNA construct encoding a target binding polypeptide according to any one of Claims 1 to 7, comprising the step of subjecting DNA encoding a target binding polypeptide to one or more cycles of mutagenesis and selection to obtain a sub-population of DNA molecules encoding target binding polypeptides having modified characteristics of affinity, specificity or avidity.

10. A method according to Claim 9, wherein the DNA encoding the target binding polypeptide is present in a replication-competent element or display vector.

11. A method according to Claim 10 wherein the display vector is selected from the group consisting of bacteriophages, filamentous bacteriophages, viruses, bacteria, yeast, slime moulds, or mammalian cells.

12. A method according to Claim 10 in which the display vector is pHFA.

13. A method according to Claim 12 in which mutagenesis is carried out using a mutator strain of *Escherichia coli*.

14. A method of producing a target binding polypeptide according to any one of Claims 1 to 7 comprising the steps of:

a) isolating DNA encoding the framework structure of one or more desired target-binding polypeptides by means of the polymerase chain reaction;

b) inserting the DNA into one or more display vectors;

c) selecting a sub-population of display vectors displaying target binding polypeptides of desired specificity, avidity or affinity;

d) subjecting the selected sub-population to one or more cycles of mutagenesis and selection in order to obtain a sub-population of display vectors displaying target binding polypeptides having modified characteristics of affinity, specificity or avidity; and

e) inserting DNA encoding the modified target binding polypeptides into a high level expression vector.

f) transferring the high level expression vector described above into an appropriate expression host,

g) expressing the target binding protein, and

h) isolating the protein thus produced.

14. A pharmaceutical composition comprising a target binding polypeptide according to any one of Claims 1 to 7, together with a pharmaceutically-acceptable carrier.

15. A diagnostic reagent comprising a target-binding polypeptide according to any one of Claims 1 to 7, together with a diluent.

16. Use of a target-binding polypeptide according to any one of Claims 1 to 7 in diagnosis.

17. Use of a target-binding polypeptide according o any one of Claims 1 to 7 as a medicament.

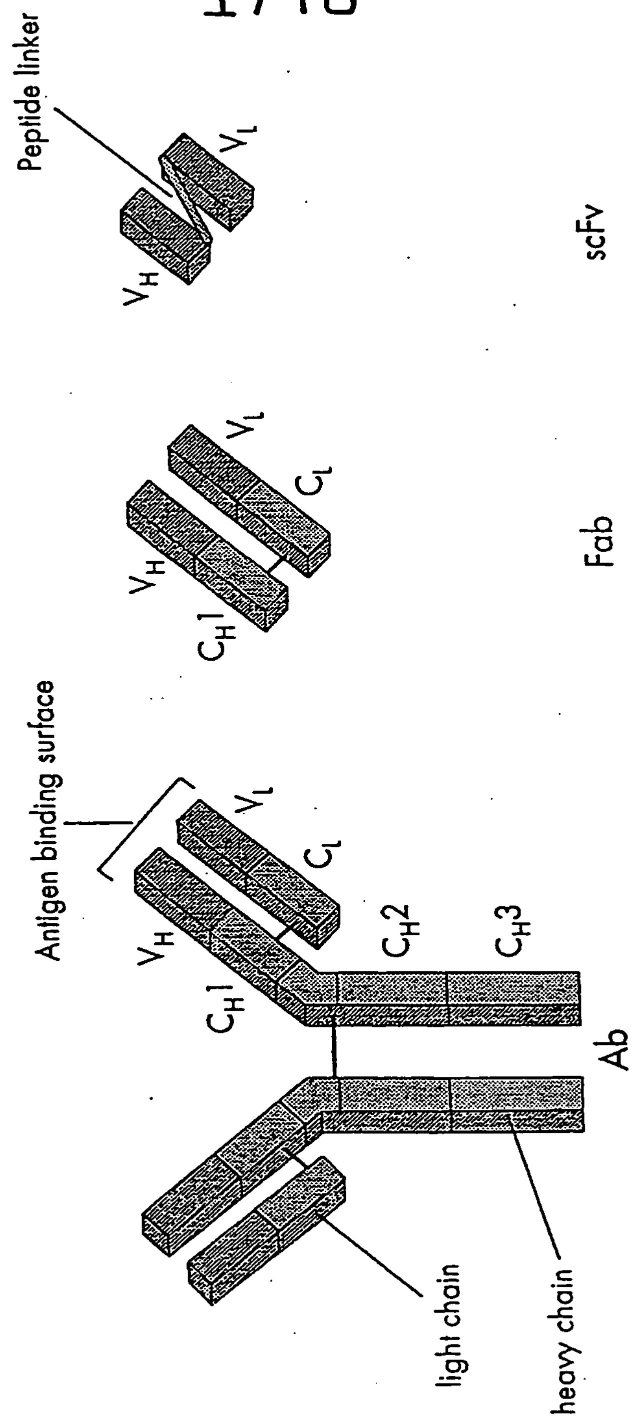
Peptide linker
V_H
V_L
scFv
V_H
V_L
C_H1
C_L
Fab
Antigen binding surface
V_H
V_L
C_H1
C_L
C_H2
C_H3
Ab
light chain
heavy chain

FIGURE 1

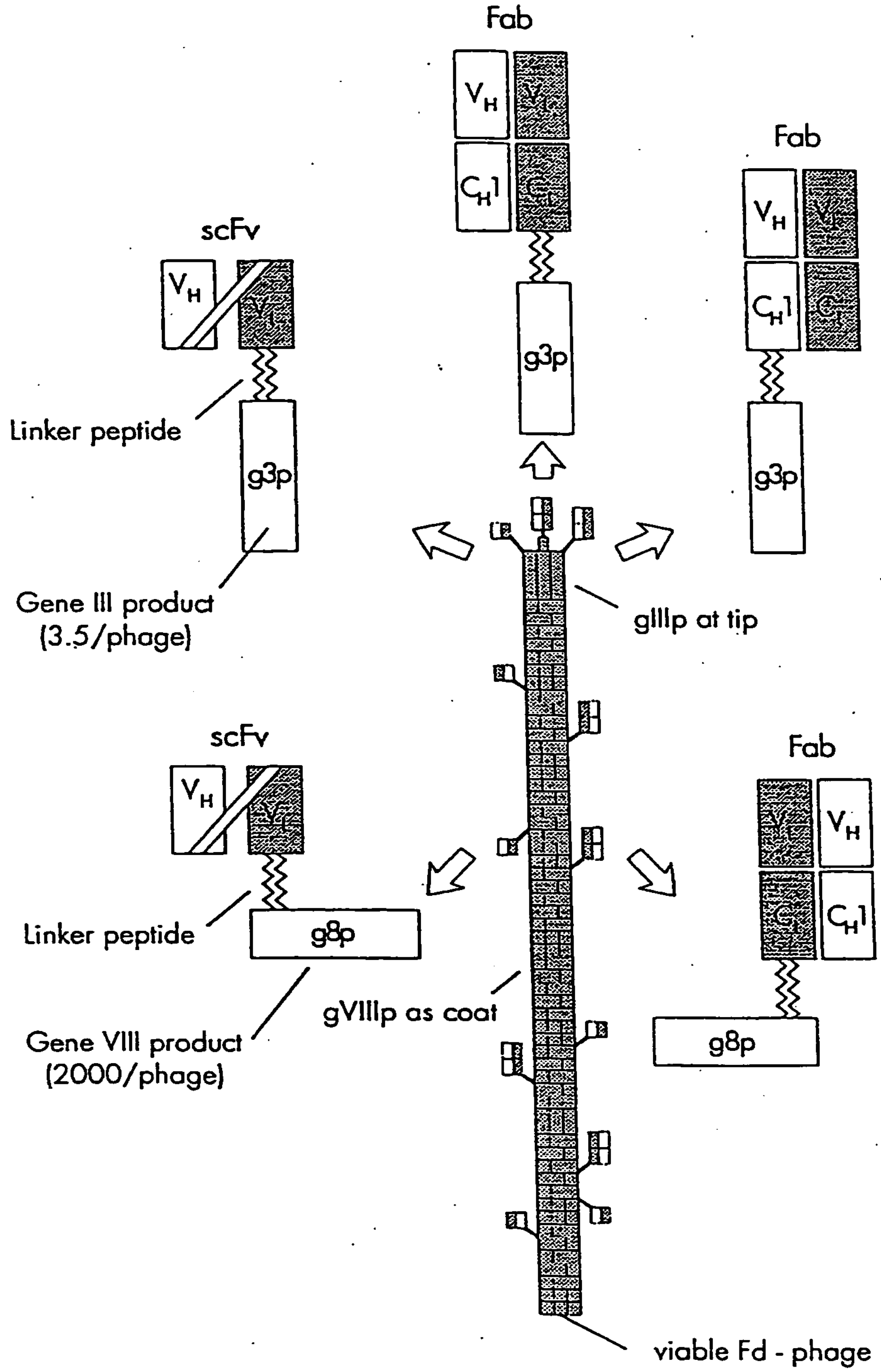
Fab
V_H
V_L
C_H1
C_L
g3p
Fab
V_H
V_L
C_H1
C_L
g3p
scFv
V_H
V_L
Linker peptide
g3p
Gene III product
(3.5/phage)
gIIIp at tip
scFv
V_H
V_L
Linker peptide
g8p
Gene VIII product
(2000/phage)
gVIIIp as coat
Fab
V_L
V_H
C_L
C_H1
g8p
viable Fd - phage

FIGURE 2

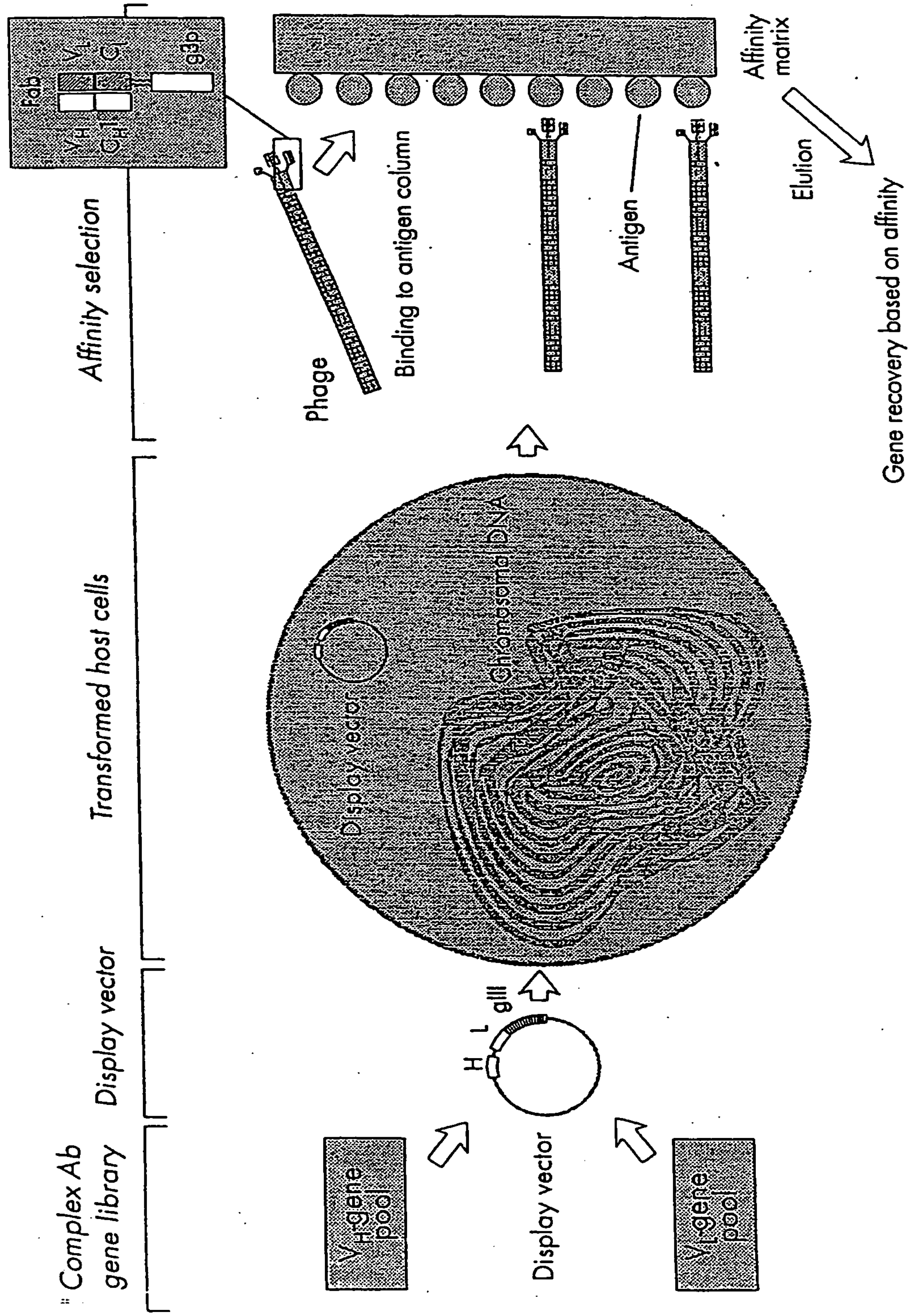
Complex Ab gene library
Display vector
Transformed host cells
Affinity selection
Fab
VH
VL
CH1
CL
g3p
Phage
Binding to antigen column
Antigen
Affinity matrix
Elution
Gene recovery based on affinity
Display vector
Chromosomal DNA
H
L
gIII
VH gene pool
VL gene pool
Display vector

FIGURE 3

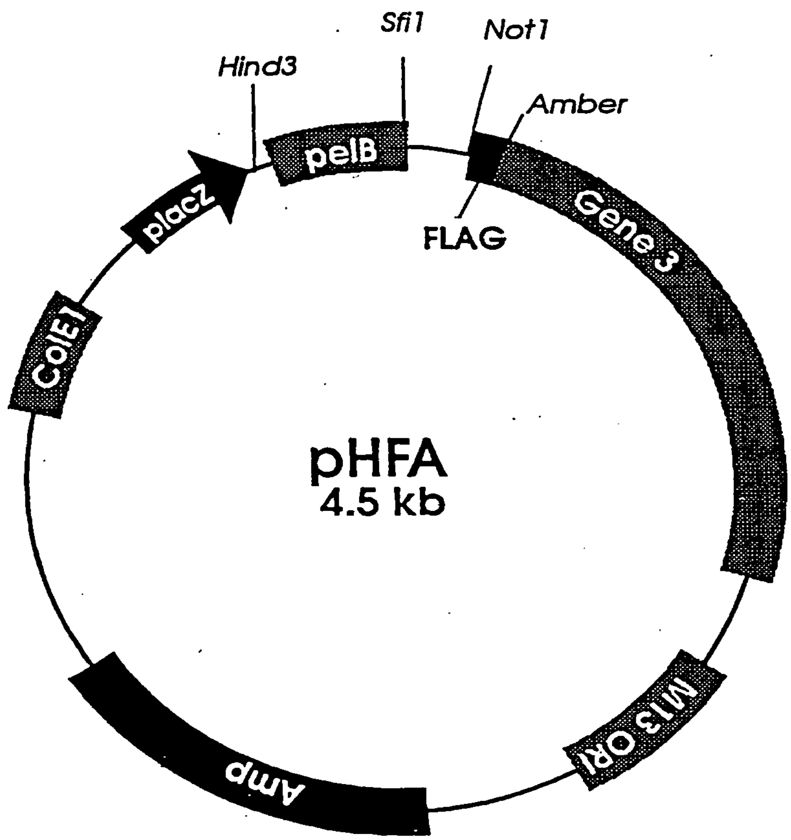
SfiI
NotI
Hind3
Amber
pelB
placZ
FLAG
Gene 3
ColE1
pHFA
4.5 kb
M13 ORI
Amp

FIGURE 4

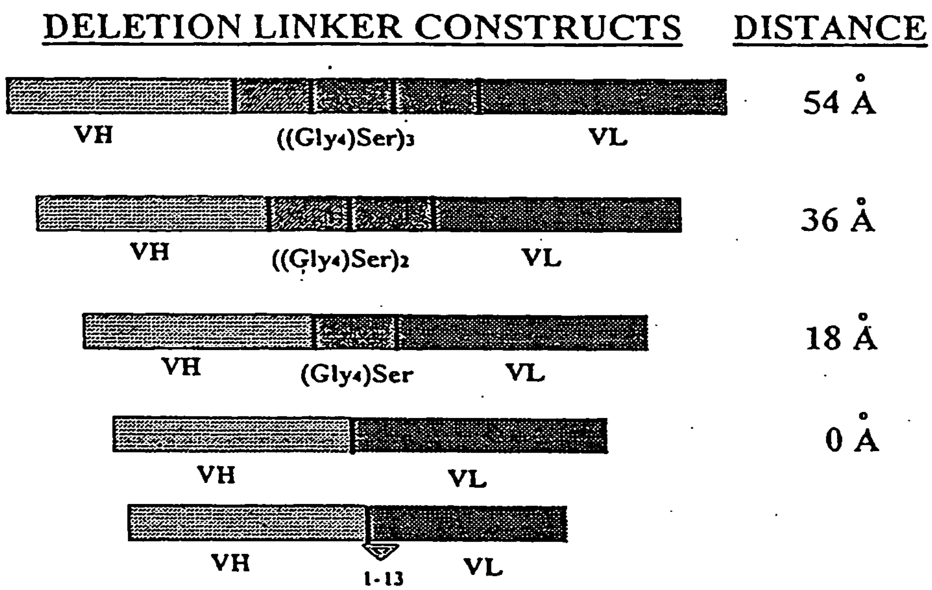
DELETION LINKER CONSTRUCTS
DISTANCE
VH
((Gly4)Ser)3
VL
54 Å
VH
((Gly4)Ser)2
VL
36 Å
VH
(Gly4)Ser
VL
18 Å
VH
VL
0 Å
VH
1-13
VL

FIGURE 5

DELETION LINKER CONSTRUCTS

Construct	Complementary Oligonucleotide Pair
$((Gly_4)Ser)_3$	5'GTC ACC GTC TCC (GGT GGT GGT GGT TCG)3 GAT ATC GAG CT 3' 3' G CAG AGG (CCA CCA CCA CCA AGC)3 CTA TAG C 5'
$((Gly_4)Ser)_2$	5'GTC ACC GTC TCC (GGT GGT GGT GGT TCG)2 GAT ATC CAG CT 3' 3' G CAG AGG (CCA CCA CCA CCA AGC)2 CTA TAG C 5'
$(Gly_4)Ser)$	5' GTC ACC GTC TCC GGT GGT GGT GGT TCG GAT ATC GAG CT 3' 3' G CAG AGG CCA CCA CCA CCA AGC CTA TAG C 5'
-	5' GTC ACC GTC TCC GAT ATC GAG CT 3' 3' G CAG AGG CTA TAG C 5'

-13.VL 5'GGG ACC ACG GTC ACC GTC TCC TCA GCC TCT CTG GGA GAC AGA GTC ACC

5' GTC GAC GAA TTC TTA TTA TTT ATC GTC ATC ATC TTT GTA GTC 3'

FIGURE 6

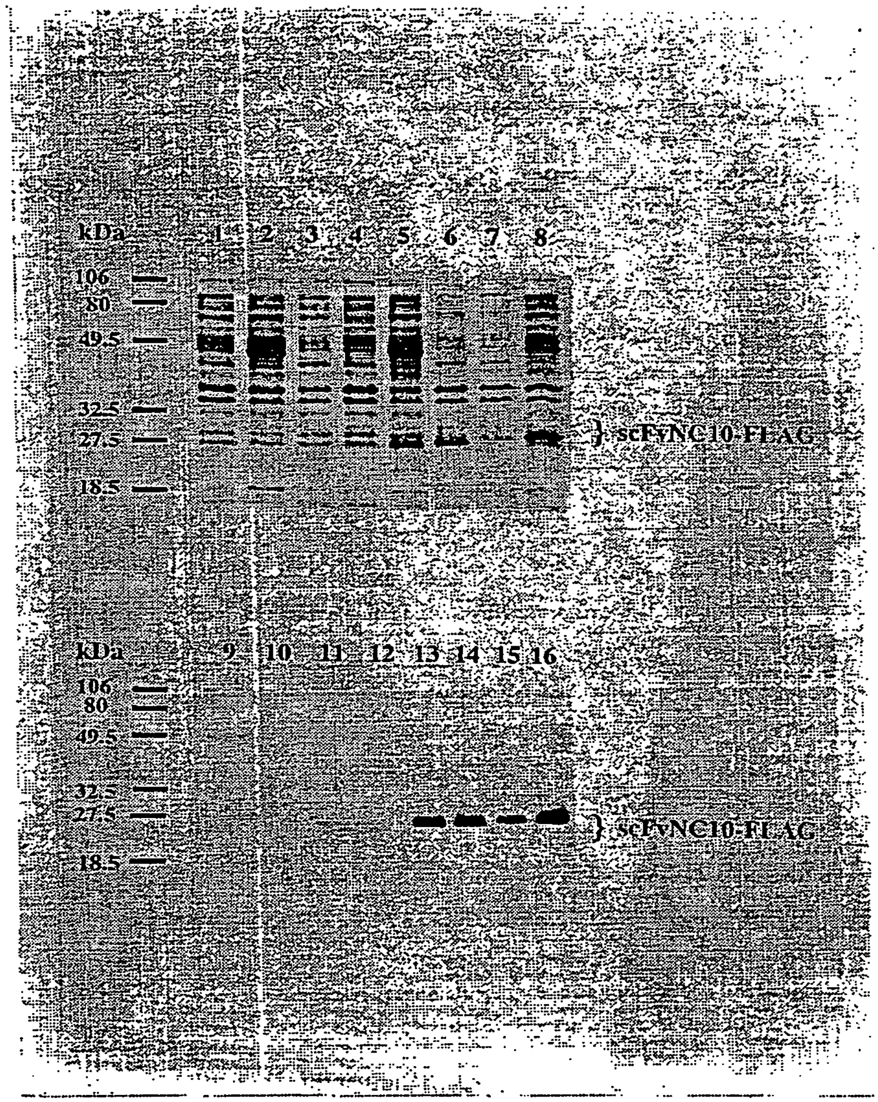
kDa
1 2 3 4 5 6 7 8
106
80
49.5
32.5
27.5
18.5
scFvNC10-FLAG
kDa
9 10 11 12 13 14 15 16
106
80
49.5
32.5
27.5
18.5
scFvNC10-FLAG

FIGURE 7

DELETION LINKER CONSTRUCTS

Linker Unit	Observed Forms	Activity
$((Gly_4)Ser)_3$	Monomers & dimers	++
$((Gly_4)Ser)_2$	Dimers	++
$(Gly_4)Ser$	Dimers	++
-	Multimers	+
-13.VL	Multimers	

FIGURE 8

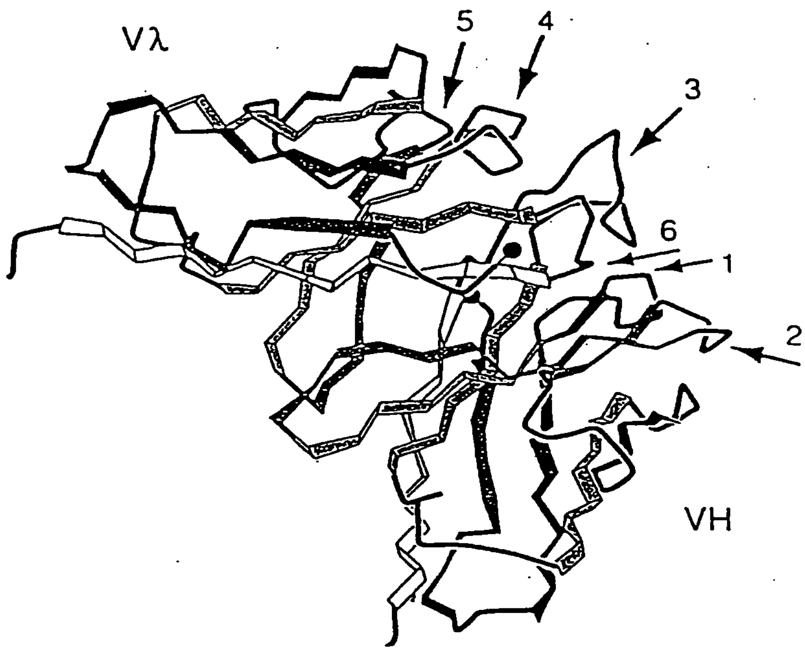

FIGURE 9a

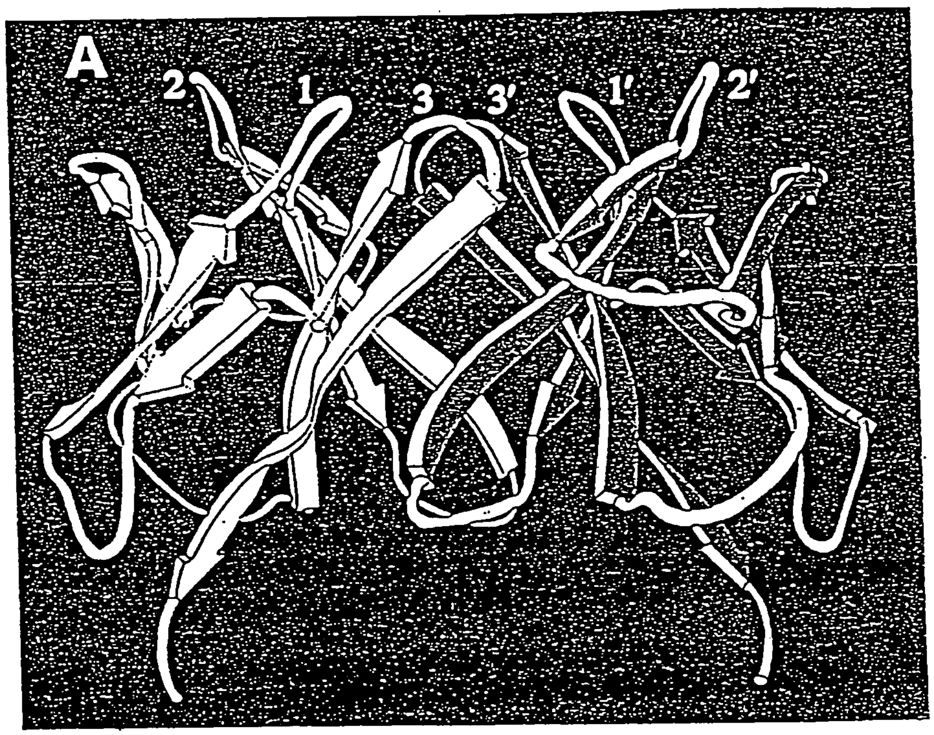

FIGURE 9b

11/ 18

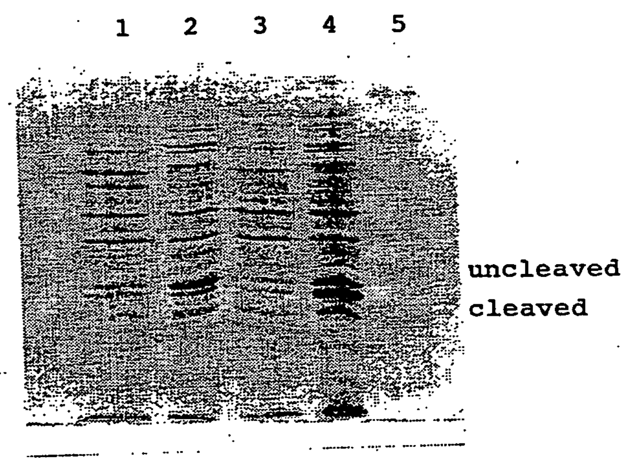

FIGURE 10

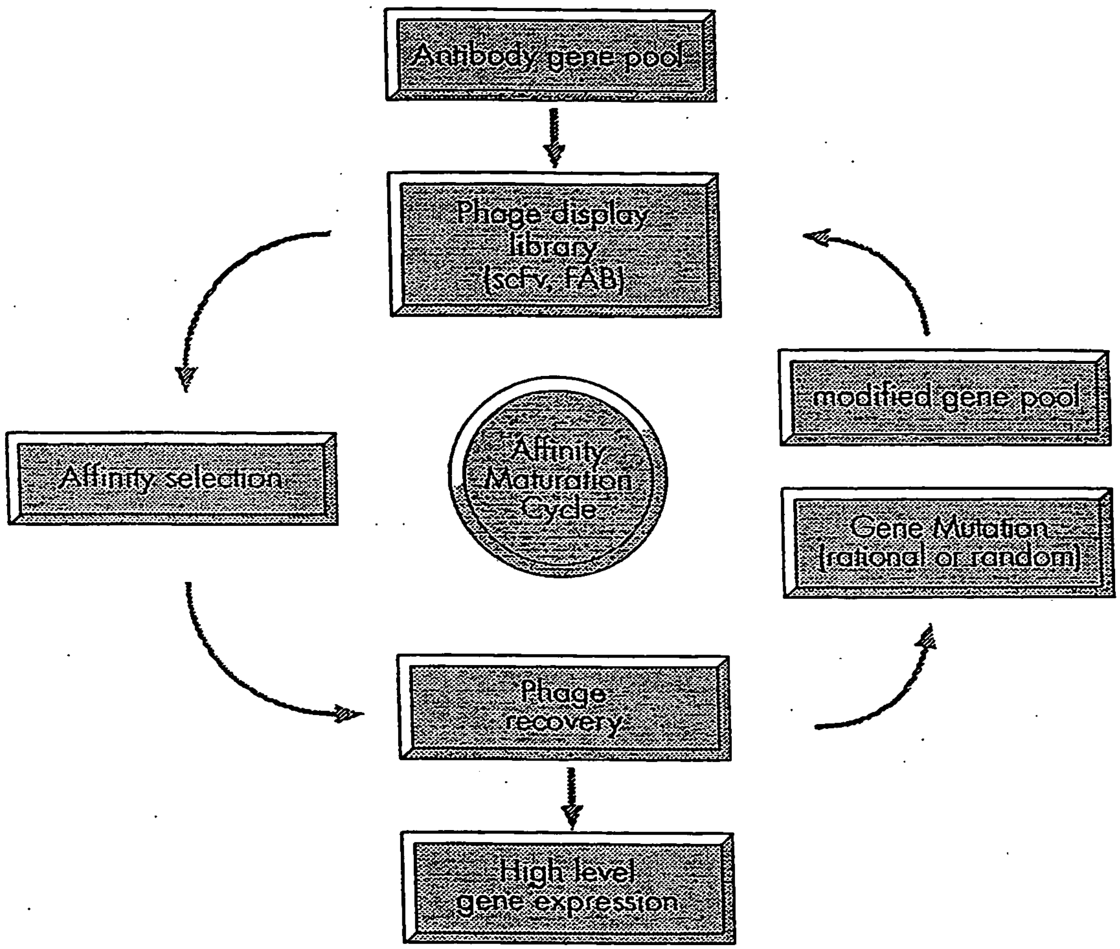
Antibody gene pool
Phage display library (scFv, FAB)
Affinity selection
Affinity Maturation Cycle
modified gene pool
Gene Mutation (rational or random)
Phage recovery
High level gene expression

FIGURE 11

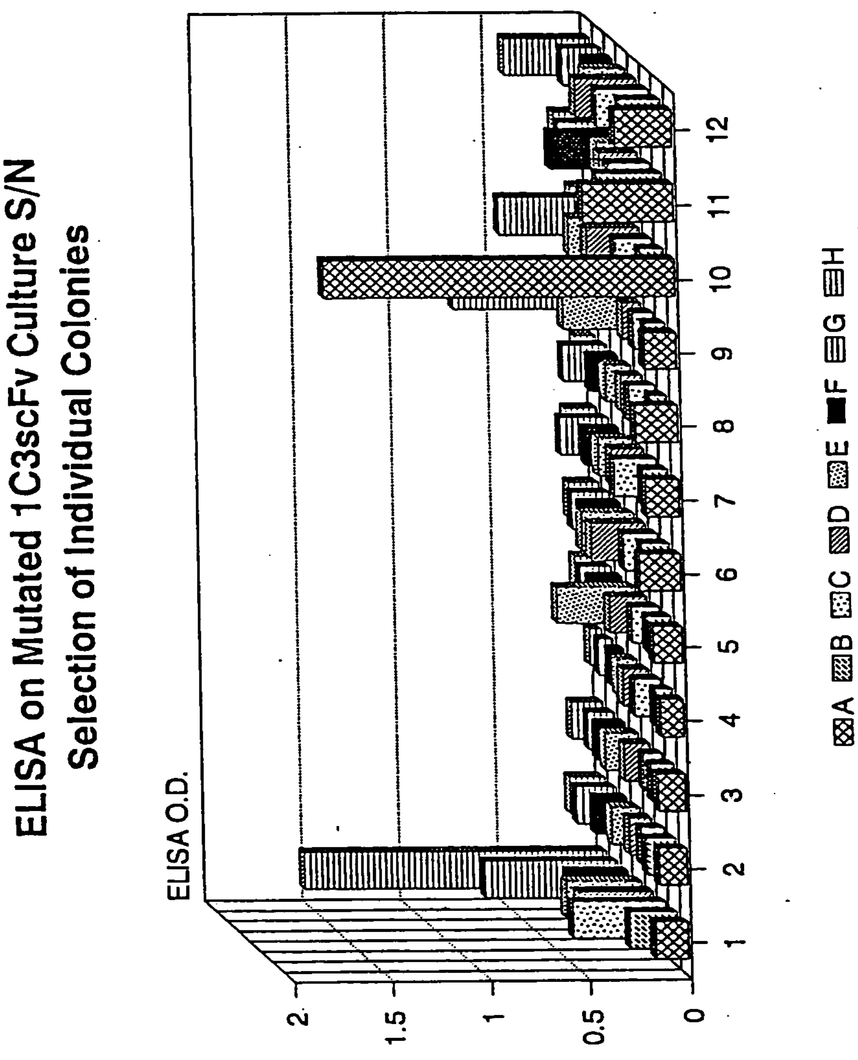
ELISA on Mutated 1C3scFv Culture S/N
Selection of Individual Colonies
ELISA O.D.
2
1.5
1
0.5
0
1
2
3
4
5
6
7
8
9
10
11
12
A
B
C
D
E
F
G
H

FIGURE 12

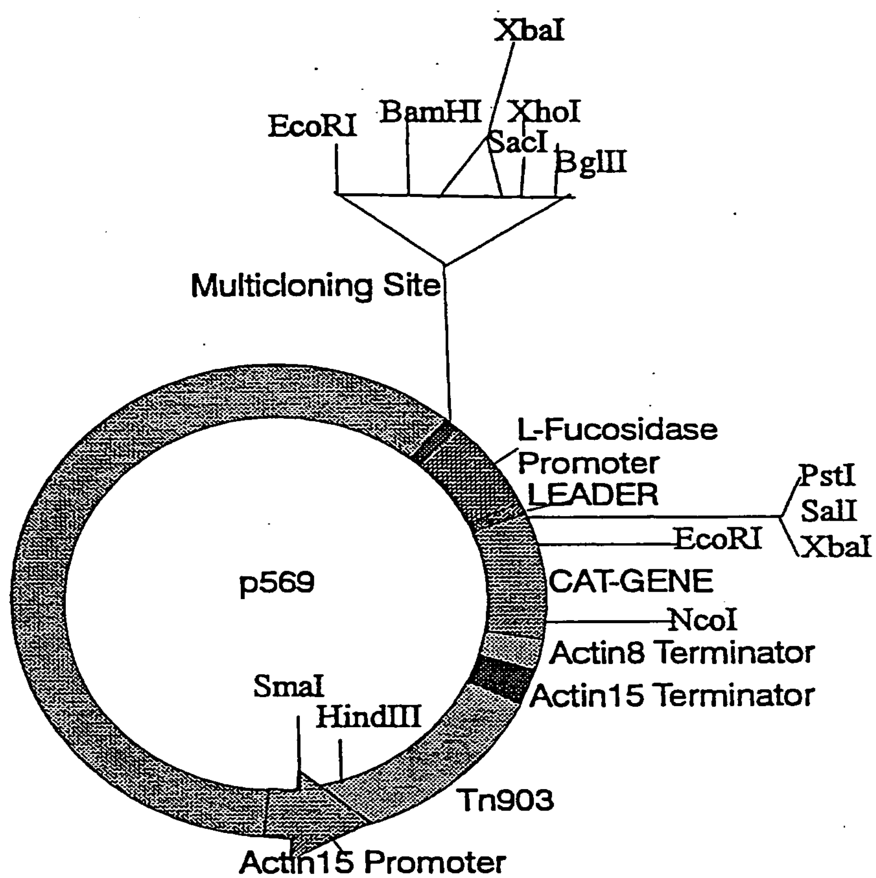
XbaI
EcoRI
BamHI
XhoI
SacI
BglII
Multicloning Site
L-Fucosidase
Promoter
LEADER
PstI
SalI
EcoRI
XbaI
p569
CAT-GENE
NcoI
Actin8 Terminator
Actin15 Terminator
SmaI
HindIII
Tn903
Actin15 Promoter

FIGURE 13

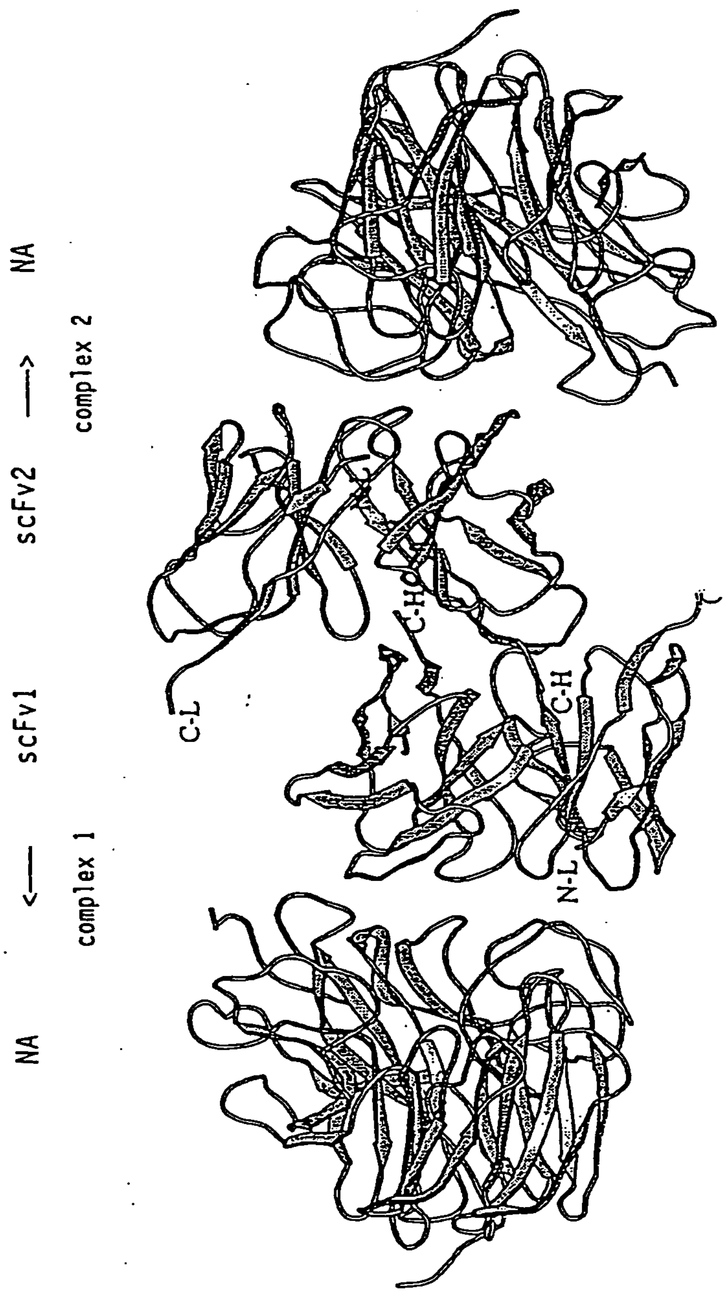

FIGURE 14

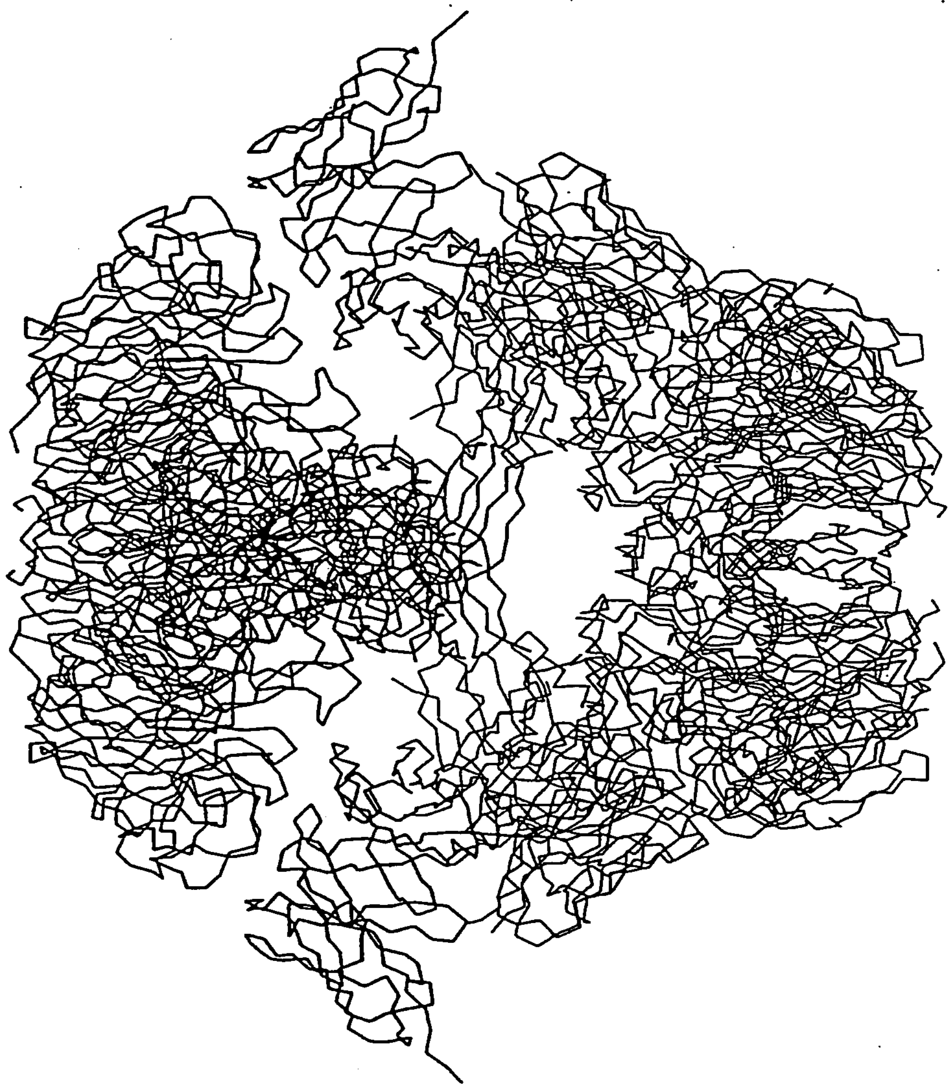

FIGURE 15

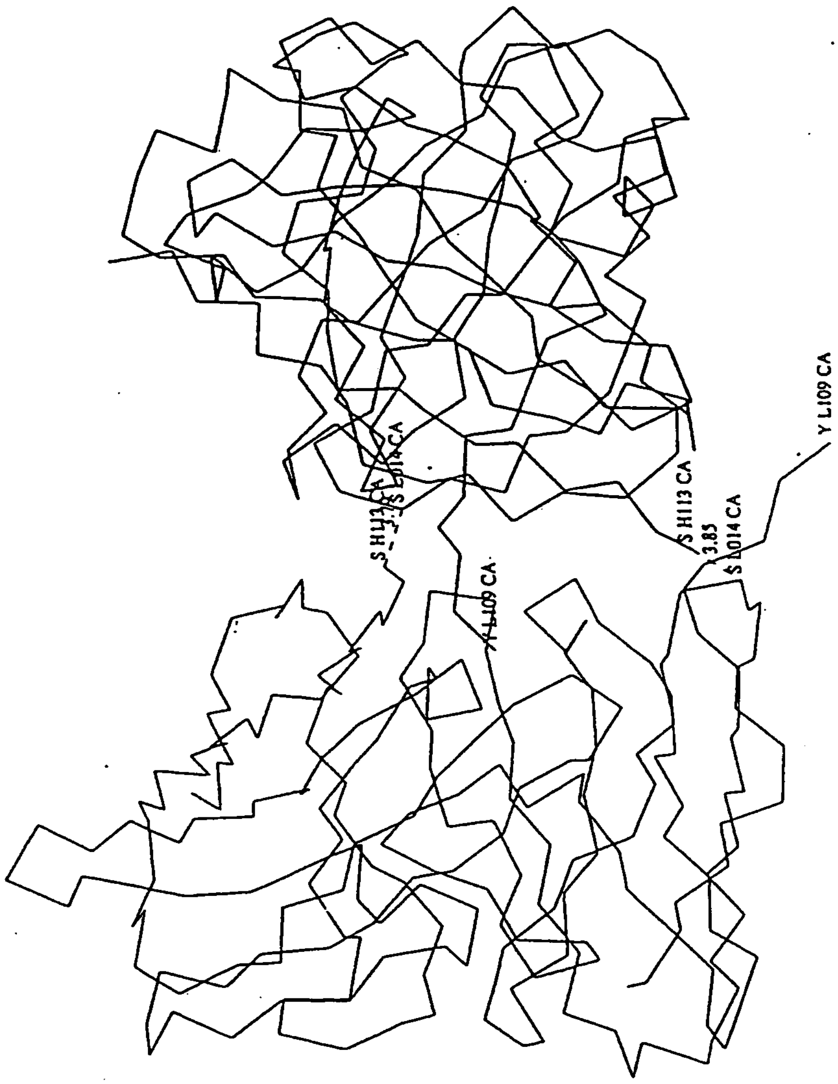

FIGURE 16

Formation of Dimeric scFvs

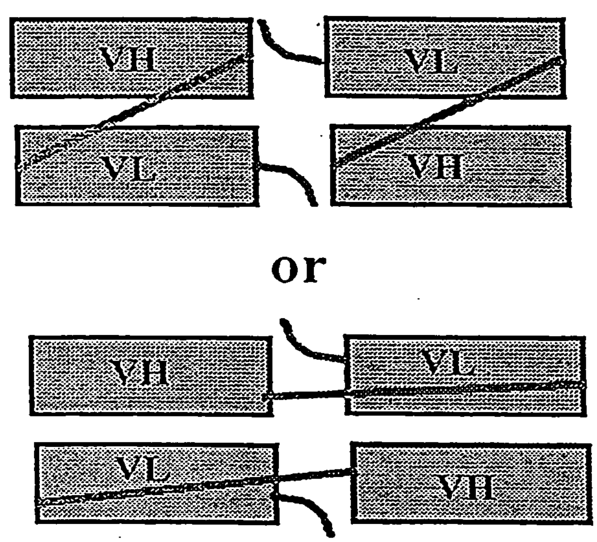

FIGURE 17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 93/00491

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.^{5} C07K 15/12; C12P 21/08; C12N 15/10; C12N 15/11; C12N 15/12; C12N 15/13

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: C07K 15/12; C07K 15/00; C07K 15/28; C07K 13/00; C12P 21/08

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC as above

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
File WPAT: Keywords: Influenza virus neurominidase; transforming growth factor alpha; leukaemia inhibitory factor; CD8
File CASA: Keywords: as above

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X,Y	Lehninger, Albert L., Principles of Biochemistry, Worth Publishers, Inc., New York, 1982. See Chapter 30, pp. 926-933.	1,2,8,9,14-17
X,Y	Erlich, Henry A. (Ed), PCR Technology, Principles and Applications for DNA Amplification, Stockton Press, New York, 1989. See Part 1, pp. 1-5	14
X	AU,A,21383/88 (Baylor College of Medicine) 25 January 1990 (25.01.90)	6

[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 January 1994 (10.01.94)	13 JAN 1994 (13.01.94)

Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. (06) 2853929	M. ROSS Telephone No. (06) 2832295

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
X,Y	Callow, Kathleen A., Measurement of Antibodies to Influenza Virus Neurominidase by an Enzyme-Linked Immunosorbent Assay. Infect. Immun. 41(2) pp. 650-6, 1983	6
X,Y	Chemical Abstracts, Vol. 113, No. 1, issued 1990 (Colombus, Ohio) Sorvillo, John, et al, Preparation and characterization of monoclonal antibodies specific for human transforming growth factor alpha, see the abstract no. 4322f. Oncogene, 5,(3), pp. 377-86, 1990	6
X,Y	AU,A,91229/91 (Immunex Corporation) 8 July 1992 (08.07.92)	1,2,5,6,8,14-17
X,Y	AU,A,79001/91 (Boehringer Ingelheim Pharmaceuticals Inc.) 27 November 1991 (27.11.91)	1-6,8-11,14-17
X	AU,B,36143/84 (596070) (The Wistar Institute) 27 June 1985 (27.06.85)	1-6,7,8-11,14-17
X	EP 239400 (Gregory Paul Winter) 30 September 1987 (30.09.87)	7

INTERNATIONAL SEARCH REPORT
Information on patent family memb

International application No.
PCT/AU93/00491

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU	91229/91	CA	20972/91	EP	561960	FI	932477
		NO	931955	WO	92/10570		
AU	79001/91	BR	9106392	EP	528951	FI	924818
		GB	9009549	HU	9203371	WO	91/16927
AU	36143/84	DE	3479289	DK	3102/85	EP	141783
		EP	291636	US	473123	US	5053224
EP	239400	GB	8607679	GB	8707252	GB	2188638
		JP	62296890	US	5225539		

END OF ANNEX